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Volume 2

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Volume 2

Edited by

MICHAEL LEDERER

Institut du Radium, Arcueil (Seine), France

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PREFACE

The success of Volume 1 of this series, as well as the numerous requests for reprints of review articles in the *Journal of Chromatography*, have convinced the editor that the publication of the reviews in book form is justified.

In discussing Volume 1 some reviewers had the erroneous notion that only some of the reviews had appeared previously in the *Journal of Chromatography*. Actually all reviews in Volumes 1 and 2 have first been published in this journal, those which were not originally in English were subsequently translated. However, owing to the rather fast development of the subject, it is envisaged to change this policy in the future and if the case should arise that insufficient space is available in the journal, some reviews might only appear in the bound volumes.

The editor will be grateful for suggestions of topics which should be reviewed as well as for review articles submitted for this series.

Thanks are due to Mr. T. J. Beckmann for assistance in the proof reading.

Paris, December 1959 — Michael Lederer

CONTRIBUTORS OF THIS VOLUME

G. BISERTE, *Laboratory of Biological Chemistry, Faculty of Medicine and Pharmacy,* Lille (France)
H. BLOEMENDAL, *Department of Biochemistry, The Netherlands Cancer Institute,* Amsterdam (The Netherlands)
J. B. HARBORNE, *John Innes Horticultural Institution,* Hertford (Great Britain)
C. J. HARDY, *Department of Chemistry, The University,* Bristol (Great Britain)
E. HAYEK, *Institute of Inorganic and Analytical Chemistry, The University,* Innsbruck (Austria)
J. W. HOLLEMAN, *Laboratory of Biological Chemistry, Faculty of Medicine and Pharmacy,* Lille (France)
J. HOLLEMAN-DEHOVE, same address
F. H. POLLARD, *Department of Chemistry, The University,* Bristol (Great Britain)
H. K. PRINS, *Central Laboratory of the Netherlands Red Cross Blood Transfusion Service,* Amsterdam (The Netherlands)
P. SAUTIÈRE, *Laboratory of Biological Chemistry, Faculty of Medicine and Pharmacy,* Lille (France)

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REVIEW OF GAS–LIQUID CHROMATOGRAPHY

C. J. HARDY* AND F. H. POLLARD

The University, Bristol (Great Britain)

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1. INTRODUCTION

During the last few years gas chromatography has become established as a rapid, efficient, and relatively simple technique for the separation and analysis of mixtures of volatile substances. The present review is intended to survey critically the large amount of published work on theoretical principles, apparatus, techniques, and applications, and to give a comprehensive bibliography on all aspects of gas–liquid chromatography.

* Present address: Atomic Energy Research Establishment, Harwell, Berks., Great Britain.

2. DEFINITIONS AND HISTORICAL

The recommendations of the Hydrocarbon Research Group of the Institute of Petroleum [580] will be followed in this review. The term "gas chromatography" will be used for all those chromatographic techniques in which a mobile gas phase carries the substances to be separated through a stationary phase packed into a suitable container. When the stationary phase is a solid adsorbent, the method is Gas–Solid Chromatography (G.S.C.); when the stationary phase is an absorbent liquid supported by inert material, the method is Gas–Liquid Chromatography (G.L.C.).

The first gas chromatographic experiments were carried out by selective adsorption on, or desorption from, solid adsorbents such as active charcoal. This basic method is of long standing, for RAMSAY [500] used it in 1905 to separate mixtures of gases and vapours. CLAESSON [101] has given a detailed bibliography of the literature up to 1944 and JANAK [328] has recently reviewed G.S.C. in detail.

Following the theoretical suggestion of MARTIN AND SYNGE [412], the method of G.L.C. was introduced by JAMES AND MARTIN [314] in 1952. This made possible the separation and estimation of small amounts of volatile substances with a very wide range of boiling points, and thus gave a great impetus to many fields of research.

3. PRINCIPLES INVOLVED IN SEPARATIONS BY G.C.

(*a*) *Methods of operating columns*

In G.C. elution analysis, frontal analysis, and displacement development can be used. For elution analysis the mixture of substances to be separated is placed at the inlet of a column of suitable granular material and is passed through the column by a carrier-gas. The components eventually leave the column in the gas stream and their concentrations are recorded as a function of the time, or of the volume of carrier-gas, by a detecting instrument. Separation of the components is achieved by repeated equilibration between the mobile gas phase and the stationary phase. Gas–solid equilibria are involved when the stationary phase is an adsorbent, and gas–liquid equilibria when it is a liquid supported on an inert granular medium. Almost any degree of separation can be achieved by varying the nature of the stationary phase and the length and temperature of the column.

In frontal analysis the mixture to be separated is carried continuously on to a column of an adsorbent and at a constant concentration in the carrier-gas. The component which is least strongly adsorbed leaves the column first and each succeeding component leaves the column mixed with a small amount of each of the preceding components.

With displacement development the mixture to be separated is adsorbed upon a column of adsorbent. A constant concentration of a substance which is more strongly adsorbed than any of the components of the mixture is then passed through the column in the carrier-gas. Each component forms a band of constant concentration

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on the column and emerges from the column substantially pure except for a small overlap fraction between each band.

Although frontal analysis and displacement development formed the basis of G.S.C. prior to the introduction of G.L.C., they have now been largely replaced by elution analysis. They are not used for analytical separations in G.L.C. (although frontal analysis has been used [61, 62] to study the symmetry of peaks) and CLAESSON [101] should be referred to for a detailed treatment of these two methods. The possibility of a form of frontal analysis or displacement development occurring during elution analysis in G.L.C. cannot be neglected, particularly where the sample enters the first section of the column, and where components of a mixture may be present at relatively high concentrations in the stationary phase. The components may then behave non-ideally and tend either to displace one another from solution, or to associate with one another and remain in solution.

(*b*) *Principles governing the elution of substances through G.L. columns*

Distinction between column efficiency and solvent efficiency

The overall efficiency of separation of a pair of solutes in a column can be divided into two contributions, (i) the column efficiency, and (ii) the solvent efficiency. The column efficiency is concerned with the spread of an initially compact zone of material as it passes through the column, and can conveniently be described by the height of a theoretical plate (HETP). The HETP is primarily a function of the column design and operating conditions, and to a lesser extent, of the natures of the solute and solvent (*i.e.* of the solvent efficiency). The solvent efficiency, or separation factor, can be expressed as the ratio of the times for two peak maxima to travel through a column, and is characterized by the respective distribution coefficients of the solutes. It is thus a function of the column temperature and of the natures of the solutes and of the solvent.

Theories have been developed to account for the rate of movement, and the characteristic shape, of zones of solutes in chromatographic columns. These theories are classified below and their application to G.L.C. first shown for a single solute. The solvent efficiency will be considered in detail later under principles governing the separation of mixtures of solutes.

Classification of theories of chromatography and their application to G.L.C.

The simplified theories of chromatography can be classified according to (i) the type of distribution isotherm that the solutes obey, and (ii) the ideality or non-ideality of the conditions. With "*linear*" chromatography the distribution coefficient (*i.e.* the amount of solute per unit volume of stationary liquid phase/the amount of solute per unit volume of mobile phase) is independent of the concentration of the solute, and with "*non-linear*" chromatography it is dependent on the concentration of the solute. In

"*ideal*" chromatography the flow of the mobile phase is considered uniform, the column is uniformly packed, equilibrium between the two phases is instantaneous, and longitudinal diffusion of solute molecules (or other processes having the same effect) does not occur. In "*non-ideal*" chromatography these assumptions cannot be made.

The four possible types are illustrated schematically in Fig. 1 (after MARTIN [405]).

1. *Linear ideal chromatography* has been dealt with in the classical treatment of WILSON [602]. The shape of the band remains unchanged during elution, mixtures

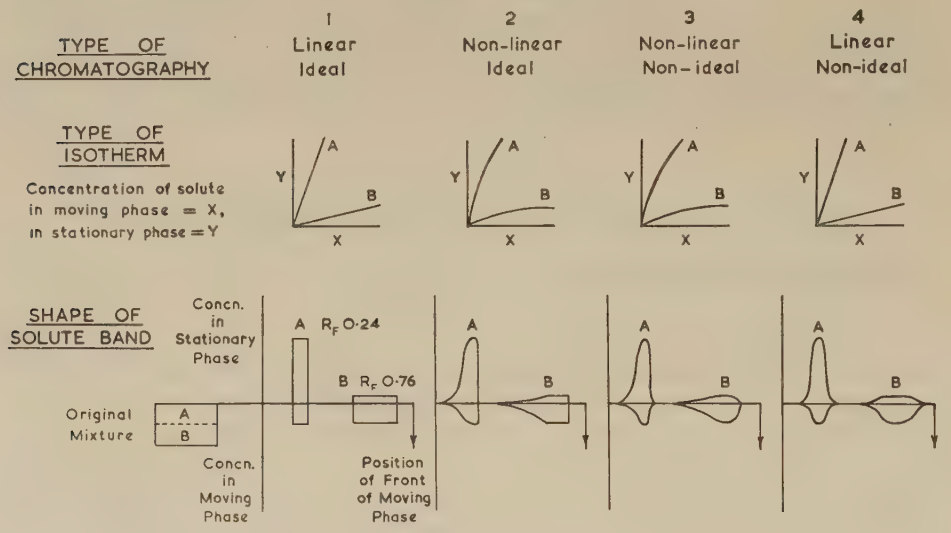

Fig. 1. Classification of types of chromatography (after MARTIN).

of solutes behave independently, and the requirements for individual bands to be separated can be calculated by simple mathematics.

2. *Non-linear ideal chromatography* was first treated by WILSON [602], and later by DE VAULT [150] for a single solute, but solutes can affect the behaviour of each other and rigorous treatment is not possible [353].

3. *Non-linear non-ideal chromatography* has been discussed by KLINKENBERG AND SJENITZER [363], but since G.S.C. is the main type of chromatography concerned and the mathematical treatment becomes very involved, it will not be considered further.

4. *Linear non-ideal chromatography* is of particular importance for the treatment of liquid–liquid and gas–liquid partition chromatography, since the assumption of a linear isotherm is usually a good approximation. The theory has been dealt with in two ways:

(i) By the "plate" theory [217, 412, 415], in which the column is regarded as a discontinuous medium analogous to a distillation column, and in which a finite volume of solution is equilibrated successively with a number of theoretical plates of solvent.

(ii) By the "rate" theory [141, 210, 219, 353, 375], in which the column is

regarded as a continuous medium in which mass transfer and diffusion phenomena are taken into account.

The shape of elution curves is given by a binomial distribution for the discontinuous theoretical plate treatment and by a Poisson distribution for the continuous treatment. Although for a sufficiently large number of equilibrations both distributions approximate to Gaussian curves, KLINKENBERG AND SJENITZER [363] have shown that their widths are different. The mechanism of the widening of a band in chromatography has been examined in terms of the "rate" theory by VAN DEEMTER *et al.* [141], who extended the theory developed by GLUECKAUF [219] and other workers. KEULEMANS [353, 357] has recently reviewed the combination of "rate" and "plate" theories, which together give a qualitative and quantitative understanding of the numerous parameters operating in G.L.C.

The three principal contributions to the broadening of a band are:

(i) eddy diffusion due to packing,

(ii) molecular diffusion,

(iii) resistance to mass transfer.

From these a basic equation can be derived [141, 357] for H, the height equivalent to a theoretical plate, in a gas–liquid column:

$$H = \underbrace{2\lambda d_p}_{\text{eddy diffusion}} + \underbrace{\frac{2\gamma D_{gas}}{u}}_{\text{molecular diffusion}} + \underbrace{\frac{8}{\pi^2} \cdot \frac{k'}{(1+k')^2} \frac{d_f^2}{D_{liq.}} \cdot u}_{\text{resistance to mass transfer}}$$

where

λ and γ	= constants,
d_p	= the particle diameter,
D_{gas}	= the gas diffusivity,
u	= the linear gas velocity,
k'	$= k(F_{liq.}/F_{gas})$,
k	= the distribution coefficient,
$F_{liq.}$ and F_{gas}	= the volume fractions of liquid and gas in the column,
d_f	= the liquid film thickness,
$D_{liq.}$	= the diffusivity in the liquid phase.

This equation is of the form $H = A + B/u + Cu$, where A, B, and C are constants referring to (i), (ii), and (iii) respectively. This is a hyperbola with a minimum H at a certain value of u at which the column is operating most efficiently (see Fig. 2). However, owing to the compressibility of the gas-phase, u is not constant over the length of the column, hence only a small section can operate at maximum efficiency. The influence of the parameters of the equation on the efficiency of separation has been discussed by KEULEMANS [353, 357], and others [140, 141, 394]. The conclusions which have been drawn are of considerable practical interest and are included later under the influence of various factors on the column efficiency.

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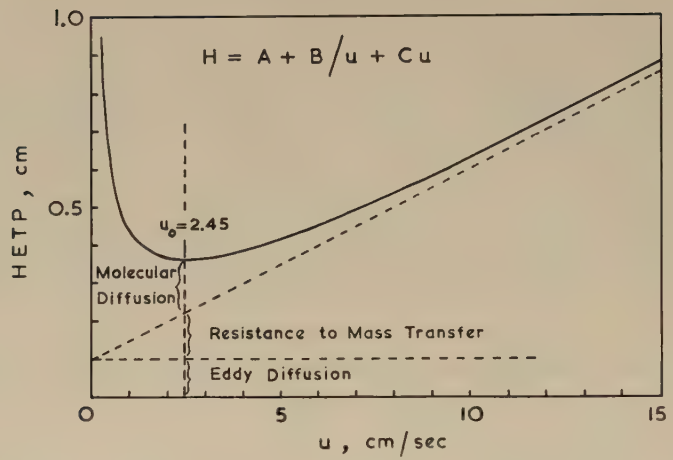

Fig. 2. Plot of HETP against calculated linear gas velocity (after KEULEMANS).

The calculation of the number of theoretical plates

Two methods were originally described [314] for the calculation of the number of theoretical plates in a gas–liquid column. Since then equations for the calculation of plate numbers from recorded chromatograms of elution peaks have been given by a number of workers [64, 395]. These equations give slightly different values and in order to provide a useful practical comparison of column efficiency, the following procedure has been recommended [580]. Tangents are drawn to the elution peak at the points of inflection. The plate number is given by $16(x/y)^2$, where y is the length of the base-line cut by the two tangents, and x is the length from the start of the elution (including the dead-volume of the apparatus) to the middle of the base-line section y.

The effect of various factors on the column efficiency (Table 1)

TABLE 1

Factor	*Effect on column efficiency (E)*	*References*
Flowrate of carrier-gas	Maximum E for a given value of linear velocity (see Fig. 2).	47, 65, 226, 353, 598, 612
Nature of carrier-gas	E greater for gases of low diffusivity and high molecular weight: $E \propto 1/\sqrt{\text{density}}$.	24, 151
Pressure of carrier-gas	E greater at (i) relatively high pressures and (ii) low ratio of inlet to outlet pressure.	65, 353
Density of packing of column	E constant above a certain density ($\sim$ 0.2 g/ml).	58, 146, 151, 334
Particle size of support	Shallow maximum E at $\sim$ 0.1 mm diameter particles.	
Amount of solvent	Optimum at $\sim$ 15–20% w/w for normal columns (see later for very high efficiency columns operated at high inlet pressure).	353, 357
Length of column	E increases with length but is not usually directly proportional.	65, 598

(Contd. on p. 7)

TABLE 1 (*Continued*)

Factor	*Effect on column efficiency (E)*	*References*
Temperature	Complex dependence of HETP on T, *e.g.*: HETP $= A + B(T) + C(T^{-1})$, which is a hyperbola with a minimum H at 24° C.	151
Sample size	E increases as size decreases.	24, 141, 486, 488
Retention volume of solute	E increases with retention volume and the empirical equation holds: log (no. of plates) = const. + ½ log (retention volume).	395, 471, 488
Conditions of sample introduction	(i) Gaseous samples give highest E if introduced in high concentration as a "plug".	353, 492
	(ii) Liquid samples introduced by a syringe give highest E if needle touches column packing; unless this occurs E depends on the temperature of injection.	486
	(iii) Liquid samples introduced in glass bulbs (or by special methods) require careful control to obtain the highest E.	353, 416

Recent developments in the construction of very high efficiency columns

Scott and Cheshire [99, 528–530] have recently re-examined the optimum conditions for the construction of very high efficiency columns. They conclude that efficiencies of the order of 900 plates per foot of column can be achieved with narrow columns containing 2.5–5% w/w of liquid phase on close-mesh (100–120 or 120–160) firebrick and operated at high inlet pressure (up to 150 lb./sq.in.) but at a low ratio of inlet to outlet pressure. The latter condition is obtained by placing a very narrow constriction at the end of the column so that the main pressure drop is across the constriction and not across the last section of the column. An example quoted for a 3.6 mm diameter column 9′ 6″ long containing 2.5% of liquid phase on 100–120 mesh firebrick operated at $p_{\text{inlet}}/p_{\text{outlet}} = 2.1$, gave plate numbers from 15,000 to 18,000 for retention times from 30–60 minutes. A mixture which was resolved into 25 components on a "normal" 1500 plate column could be resolved into 50 components on the 15,000 plate column. It is notable that, at the maximum inlet pressure that the apparatus normally withstands, the HETP was still decreasing markedly with increasing flowrate and it is expected that operation at still higher pressure and flowrate should lead to lower values of HETP (*i.e.* < 0.3 mm).

Scott predicts that 100,000 plate columns can quite feasibly be constructed based upon the above conclusions and provided that a small sample and a highly sensitive detector are used.

(*c*) *Principles governing the separation of mixtures of substances*

The forces of interaction between solute and solvent

In the choice of a stationary phase (solvent) which will give large separation factors between the solutes to be separated, the forces of interaction between solutes and solvent are of great importance. The forces can be divided into four types [359]:

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(i) Dispersion, London, or non-polar, forces arising from synchronized variations in the instantaneous dipoles of two interacting species; these are present in all cases, and are the only sources of attraction energy between two non-polar substances.

(ii) Induced dipole, or Debye, forces resulting from the interaction between a permanent dipole in one molecule and the induced dipole in a neighbouring molecule; these forces are usually relatively small.

(iii) Orientation, or Keesom, forces resulting from the interaction between two permanent dipoles, the association energy depending upon both the sizes and positions of the dipoles. (The "hydrogen-bond", *i.e.* the relatively strong interaction between negatively charged atoms and positively charged hydrogen in groups such as OH, NH, and FH, is a particularly important type of orientation force and is of considerable practical use in G.L.C.)

(iv) Specific interaction forces, and chemical bonding, *e.g.* complex formation between solutes and metal ions, or loose adduct formation between solutes and solvents.

These forces of interaction between solutes and solvents govern the relative volatilities of the solutes and therefore the separation achieved. With a non-polar solvent, non-polar solutes will tend to be separated approximately in the order of their boiling points since the forces of interaction (dispersion forces) between solutes and solvent will be similar to the forces between the solute molecules themselves. Polar solutes will be eluted from a non-polar solvent more rapidly than non-polar solutes of a similar boiling point, as they will have lost the strong dipole–dipole association energy which is present in their own liquid [471]. As the polarity of the solvent is increased the polar solutes will be retarded to a greater extent. Solvents of different polarities have been used by a number of workers [63, 316, 359, 395, 570] to effect the separation of mixtures of solutes such as aliphatic, naphthenic, olefinic, and aromatic hydrocarbons. Olefin selectivity increases in a series of solvents of increasing polarity [359], *e.g.* hydrocarbon oil–dinonyl phthalate–dibutyl phthalate–ethyl acetoacetate–dimethylformamide.

By comparing the behaviour of solutes in two or more solvents it is often possible to determine (i) the chemical type and (ii) the position of the solutes in their homologous series. James and Martin [316] examined the behaviour of a series of aliphatic compounds, C_5H_{11}–X, and found that the elution sequence in an aromatic solvent, benzyldiphenyl, is (increasing interaction):

$$X = H < Cl < Br < I < NH_2 < NO_2 < OH < COCH_3 < CN,$$

while in liquid paraffin or a poly-ether the sequence is:

$$X = H < OCH_3 < Br,Cl < I < NO_2 < COCH_3 < NH_2 < CN < OH.$$

The authors stress the usefulness of these sequences, as well as the regular behaviour within homologous series, for the determination of unknown substances.

The retardation of polar solutes can be made very large if H-bonding can take place. A clear illustration of this effect is provided in the early work of James and Martin [313] on the separation of primary, secondary, and tertiary amines using as

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solvents liquid paraffin, and Lubrol MO (which contains a poly-ethylene oxide structure). The Lubrol MO retards primary and secondary amines due to H-bonding, but does not retard tertiary amines. Comparison of the behaviour of mixtures of amines on these two solvents allows the chemical types of the amines to be identified.

Specific interaction forces probably partly account for the strong retardation of aromatic hydrocarbons by aromatic solvents, *e.g.* the 1:1 picric acid–fluorene addition compound has been recommended [359, 595–6] as a solvent with very high selectivity towards aromatic solutes. A solution of silver nitrate in glycol or other solvents has been shown [37, 63, 113, 212, 391, 473] to retard unsaturated hydrocarbons which interact specifically with the silver ion to form weak complexes. Metal salts of fatty acids have recently been proposed as solvents with a high selectivity towards amines [473, 475], and amino-acid esters [33]. These open up a new field in which use can be made of the interaction forces between solutes and metal ions or complexes.

Classification of solute and solvent type

Although the existing theories of solutions do not allow the separation of given solutes by a given solvent to be predicted in more than a few simple cases, a qualitative scheme of classification has been put forward [353]. Five classes of substances are defined in order of decreasing cohesive energy, and a solute will be retained more strongly by a solvent as the classes to which they belong are closer together. However, the classification does not explicitly take into account the effects of mutual polarisability, nor does it allow for the effect of forces, such as those of the chemical bond, which may lead to strong retention of particular solutes by certain solvents.

(I) The molecules form a three-dimensional network of hydrogen bonds. Examples are: water, poly-alcohols, amino-alcohols, oxy-acids, polyphenols, di- and tri-carboxylic acids.

(II) The molecules possess active hydrogen atoms as well as electronegative atoms with free pairs of electrons (O,N,F). Examples: alcohols, fatty acids, phenols, primary and secondary amines, oximes, nitro-compounds and nitriles with α-H atoms.

(III) The molecules possess electronegative atoms but no active hydrogen. Examples: ethers, ketones, aldehydes, esters, tertiary amines, nitro-compounds and nitriles without α-H atoms.

(IV) The molecules possess active H-atoms and negligible dipoles only. Examples: $CHCl_3$, CH_2Cl_2, CH_3CHCl_2, $CH_2Cl \cdot CH_2Cl$, etc., aromatic and olefinic hydrocarbons.

(V) Molecules without functional groups, such as saturated hydrocarbons, carbon disulfide, tetrachloromethane, etc.

The effect of temperature on the solvent efficiency

Since the separation of solutes depends upon the distribution of molecules between the gas and liquid phases, corrected retention volumes (V_R^0, the volume of mobile phase required to elute the maximum of a peak and corrected for dead-volume of column, pressure-drop across column [580], and measured at the column temperature)

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vary with the temperature according to a Clapeyron-Clausius equation. For small variations of temperature

$$\frac{\mathrm{d} \ln V_R^0}{T} = \frac{\Delta H_S}{RT^2} + K'$$

where

ΔH_S = the heat of solution of the solute in the solvent,
K = a constant for different solutes in a given solvent.

The heat of solution is negative (*i.e.* heat evolved) and because of the increasing solute–solvent interaction usually increases numerically with the retention volume.

If V_1 and V_2 are the retention volumes of solutes S_1 and S_2 ($V_2 > V_1$), the separation factor (V_2/V_1) will decrease as the temperature is raised since

$$\frac{\mathrm{d} \ln V_2/V_1}{\mathrm{d}\, T} = \frac{\Delta H_2 - \Delta H_1}{RT^2}$$

In a plot of log V against $1/T$ approximately straight lines are obtained (indicating that this type of equation is obeyed) and the lines for V_1, V_2, V_3, etc. tend to be closer together at high temperatures than at low temperatures (hence smaller separation factor). As a general rule therefore, better separation factors are obtained by operating at low temperatures.

The presentation and correlation of G.C. data

Gas-chromatographic data have been presented in many forms, *e.g.* as retention times, or volumes, relative to various arbitrary standard solutes, and as absolute retention times, or volumes. The committee appointed to investigate the presentation of data has recommended [11, 580] that one of the following should be given (for definitions see below):

(i) *The specific retention volume* (V_g) *or the partition coefficient* (k) for at least two temperatures (if only two then as far apart as possible). The value of V_g or k may be given relative to a standard solute, particularly if this gives a straighter relationship between log V_g and $1/T$.

(ii) An equation relating V_g, or k, and temperature, for example

$$\log V_g = \frac{\Delta H_s}{2.3\, RT} + D,$$

or an Antoine equation,

$$\log V_g = \frac{A}{t + B} + C,$$

where

V_g = the retention volume fully corrected for pressure-ratio across the column, dead-volume of apparatus, sample size, and weight of solvent,
k = the weight of solute per ml solvent ÷ weight of solute per ml of gas phase,
ΔH_s = the heat of solution,
T and t = the temperature in °A and °C respectively,
A, B, C and D = constants determined to fit the equations.

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Examples of these methods of presentation are given by YOUNG [613] and AMBROSE [10]. The use of punched cards has been described [547, 603] for the storage and reporting of G.C. data, and this will probably become widely used.

The committee has recommended [580] standard solutes and solvents (see p. 20) and further considers that it is important to include in published work the following: the nature of the support, the nature and weight of the solvent, the sample size (if not corrected for), inlet and outlet pressures, the method of flow-measurement, the accuracy of temperature control, the type of detector, and the density of the solvent (if known) since this enables V_g and k to be interconverted.

Several empirical methods have been used for correlating gas-chromatographic data for solutes in homologous series, and for solutes of similar structure, with other physical data. Straight lines are usually obtained for plots of the log retention volume (or relative retention volume) in a given solvent against:

(i) The number of carbon atoms in the molecule [314, 351, 353, 501],

(ii) The boiling point of the pure solute [180, 235],

(ii) The log of the vapour pressure of the pure solute [283, 284, 496],

(iv) The inverse of the solvent temperature [353, 395, 489],

(v) The log of the retention volume (or relative retention volume) in a second solvent [476]; alternatively a non-log plot can be used [305, 319].

Data in the above form can be used for the qualitative analysis of quite complex mixtures (plots iv and v), for the determination of thermodynamic properties, *e.g.* heats of solution (plot iv, see later for details), and for the determination of retention volumes of solutes (a) for which no standards are available (extrapolation of type i plots) or (b) at temperatures other than the measured ones (type iv plots).

HERINGTON in his thermodynamic treatment [281] of G.L.C. as an example of extractive distillation, deduced the fundamental relation for a pair of solutes in a homologous series:

$$\log V_{21} = \log (p_1^0/p_2^0) + \log (\gamma_{13}^0/\gamma_{23}^0)$$

where

V_{21} = the retention volume of solute 2 divided by that of solute 1,

p_1^0 and p_2^0 = the vapour pressures of the pure liquids 1 and 2 at the temperature employed,

γ_{13}^0 and γ_{23}^0 = the activity coefficients of solutes 1 and 2 at *infinite dilution* in the binary mixtures 1, 3 and 2, 3 respectively, where component 3 is the solvent.

Examination of the various terms in this equation using experimental data other than those from G.L.C. reveals the theoretical foundation for many of the empirical relations in current use, *e.g.*, plots of types (i)–(v) above.

Activity coefficients (preferably at infinite dilution γ^0) can be relatively easily calculated from gas-chromatographic data (for details see later), and since they are much less dependent on temperature than are retention volumes or partition coefficients, they can conveniently be used for the characterization of solute–solvent systems.

References p. 32/43.

TABL

CROSS-REFERENCE OF CLASSES OF SOLUTE

The bibliography references to the original papers in which a particular class of solute has been separate

General class of solvent	Examples of solvents	Class of substance separated: Saturated, unsaturated, aromatic, etc., hydrocarbons <C10	Hydrocarbons >C10	Halogenated hydrocarbons	Volatile fatty acids and their esters	Alcohols
Hydrocarbons	*n*-hexadecane (C16)	353, 357, 373		505, 513	373	373
	benzyldiphenyl	149, 316, 319		316	316	316
	squalane	160, 179–181, 373, 425, 476, 492, 570, 617			570	476
	liquid paraffin	28, 113, 151, 172, 316, 496, 512, 526–7, 592–4		28, 254, 316, 488, 497, 498, 596	28, 596	316, 596
	n-hexatriacontane	148–9				
	tri-isobutylene	30, 63, 254				
	fluoro-hydrocarbons			187, 505		
Alcohols	glycerol			10, 488–9		85
	polyethylene glycols	2, 13, 57, 58, 73, 605		187	2, 333–4, 596	2, 52, 58, 137, 333, 596
Ethers	Lubrol MO	318				
Esters	dinonyl phthalate	28, 51, 65, 73, 85, 123, 129, 249, 353, 359, 435, 501–2, 596		10, 36, 186–7, 249, 250, 254, 486, 488–9, 511, 519, 576	28, 487, 501, 511, 596	1, 28, 123, 486, 501, 596
	didecyl phthalate	160, 201, 230, 364, 476, 492, 556		160, 258		160, 258, 476, 492
	other alkyl phthalates	280, 326, 359, 374, 390, 455, 496, 518, 544, 556		187, 238, 465, 488		353
	tricresyl phosphate	83, 241, 379, 523, 566		241, 254, 255, 566	379, 395, 418	379, 395, 523
	tritolyl phosphate	10, 259, 395		259	10, 325, 395	10, 259, 395, 469
	fluorene picrate	595–6				
Silicone and other oils and greases	silicone 702 or 703	10, 249, 250, 395, 473		10, 187, 250, 488–9	10, 325, 395	10, 395, 473
	silicone 550, etc.	156, 440, 469, 538, 595–6, 614	596, 614	188, 231, 254, 366, 417, 488	16–19, 304, 313–4, 334, 343, 418, 469	469, 614
	silicone high vac. grease	51, 429	3, 127	187, 429	3, 38, 117–119, 127, 159, 297, 545, 566	118, 119, 159, 351
	Apiezon L	528	3, 527		3, 38	
	Apiezon M or others	99, 158	336	187	158, 351, 378, 590	
Metal salts	$AgNO_3$/glycol, etc.	37, 63, 106, 113, 212, 391, 473	106			
	metal stearates, caproates, etc.	473				473
Miscellaneous	polythene		127		38, 127, 351	
	sugars					
	dimethylformamide	113, 353, 356, 359, 440, 518, 568, 605				
	dimethylsulpholane	113, 182, 201, 277, 353, 364, 558				
	zeolites and alkyl-ammonium bentonites	31, 327, 592–4, 597		327		31
	nitrobenzene	518		202		

It is not important for the presentation of results whether γ^0 or k^0 (the value of k at infinite dilution) is used, since γ^0 can be calculated from p^0 and k^0 and vice versa. However, γ^0 is particularly useful for discussing solute–solvent interaction and for correlating the structures of solutes and solvents with their chromatographic behaviour. PIERROTTI and co-workers [476, 492] have developed a method (PIERROTTI's "Building Block" Method) for correlating activity coefficients with the structures of the solute and the solvent. They have determined activity coefficients prevailing in a large variety of binary mixtures, so chosen that variations within homologous series of both solutes and solvents could be studied systematically. Although much of this work has involved solvents of volatilities too high to be of direct interest for G.L.C., certain regularities in behaviour are applicable to the understanding, and prediction, of the behaviour in practical G.L.C. systems (for example, see the discussion by KEULEMANS [353]).

References p. 32/43.

RATED BY A NUMBER OF SOLVENTS

particular solvent are given at the intersection of the respective horizontal and vertical columns.

	Class of substance separated									
etones	Aldehydes	Ethers	Sulphur-containing compounds	Nitro-compounds and organic nitrates, etc.	Amines and amino-acid derivs.	Boron, silicon or deuterium compounds	Essential oils, terpenes, etc.	Pyridine bases	Phenols and derivs.	H_2O
	373		149		307					
570		570								
			512, 527		301, 307, 319	346(B)		74, 313		596
			149							
					313			74, 76, 445		445
2, 57, 137, 596		52	137					74, 76		2, 52, 596
					301, 307, 319			313		
85, 123, 429, 501, 596, 613	85, 123, 501	64, 65, 501, 558	546	75, 249, 250, 487				74, 76		1, 123
258, 476	160					604(D)				
			557	490					344–5	
	379		7, 523		395	346(B)	384			
	259	259, 469				346(B)				
488		473			473					
	294				319		167, 384	74, 76, 313		
	159				33, 34		508			
			270, 566						270, 297	
508		95					289, 290, 508		95	
		473			33, 34, 473, 475					
									329	
		558								
						202(Si)				

Analytical applications of G.L.C.

A very wide range of substances have now been examined by G.L.C. and examples of the solvents used to separate various classes of substances are illustrated in Table 2. In principle all components of mixtures which can be separated (and usually qualitatively analysed) can also be quantitatively analysed by the application of methods suitable to the detector employed, and which are reviewed in Section 4.

References to the application of G.L.C. to the complete or partial analysis of some complex mixtures are given below.

Organic fluoro-compounds [21, 185–7, 221, 238, 465, 513–5, 519, 541, 553, 559, 563].

Combustion products of internal combustion engines [88, 148, 182, 195, 272, 295].

Essential oils [41–2, 49, 66, 87, 167, 288–291, 300, 387, 446–8, 468, 508, 552, 569, 588, 609].

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Components of tars [72, 234–5, 297–8, 344–5] and tar heavy oils [175].
Naphthalene hydrogenation products [544].
Trace analysis at parts per million level [40, 56, 126–7, 272, 589].
Solvents for plastics and lacquers [126, 259, 260, 372, 378, 596].
Determination of steroid side-chains [111].
Monitoring of distillates from fractional distillation columns [221, 438, 487].
Halogen and interhalogen compounds [184].
Flavours in fruit, vegetables, and other foodstuffs [96, 110, 127, 161–4, 189, 262, 292, 300, 333, 380, 437, 506, 539, 540, 551, 588].
Components of tobacco smoke [460, 464, 498, 532, 534].
Products of Fischer-Tropsch reactions [129].
Products of reaction-kinetics studies [84–5, 104, 123, 170–2, 209, 348, 366, 422–5, 490].
Separation of *o*-, *m*- and *p*-nitrotoluenes [451], and *m*- and *p*-xylene [200, 374, 458].
Volatile fatty acids (and their esters) in natural products [16–19, 24, 38, 109, 115, 117–120, 266–9, 274–5, 296, 311–7, 320–2, 343, 351, 385, 386, 392, 418–420, 453–4, 462, 485, 498, 536–7, 572, 581, 590].
Polymer degradation products [135] and impurities [126, 560, 587, 606].
Insecticides and antioxidants [3, 332].
Radiolysis products of organic compounds [83, 152–3, 165, 188, 195, 230, 507, 600].

(*d*) *The determination of activity coefficients and heats and entropies of solution by G.L.C.*

A number of recent papers [12, 14, 284, 353, 373, 395, 492, 593] have shown the usefulness of G.L.C. in the study of the thermodynamics of systems involving volatile solutes and involatile solvents. Activity coefficients and free energies of solution are derived from retention volumes at a given temperature; heats of solution are derived from the temperature dependence of the retention volume; and entropies of solution can be derived from the combination of heats and free energies of solution. KEULEMANS [353] reviewed the methods for the calculation of γ^0, the activity coefficient at infinite dilution, from symmetrical and asymmetrical elution curves and tabulated values of γ^0 for a number of hydrocarbons and oxygenated compounds. However, the data were not regarded as highly accurate since they were derived from incidental analyses carried out for other purposes. A greater accuracy can be achieved in specially designed experiments, for example, the work of KWANTES AND RIJNDERS [373]. These authors also measured values of γ^0 for polar solutes in non-polar solvents by using fine metal helices as the inert support, and extended the method to solvents of relatively high volatility by presaturating the carrier-gas with solvent vapour. In this way, the value of γ^0 for a normal hydrocarbon in its next higher homologue was determined with fair accuracy.

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The determination of the activity coefficient at infinite dilution, γ^0

The value of γ^0 is usually obtained in the form of $\gamma_p{}^0$ or $\gamma_f{}^0$ from the equations:

$$\gamma_p{}^0 = \frac{N_{11q.}\,RT}{k.p^0} \quad \text{or} \quad \gamma_f{}^0 = \frac{N_{11q.}\,RT}{k.f^0}$$

where

p^0 and f^0 = the vapour pressure and fugacity respectively of the solute at temperature T°A,

$N_{11q.}$ = the number of moles of solvent per unit volume at temperature T,

k = the partition coefficient derived from:

$$V_R^0 = V_{gas} + k\,V_{11q.}$$

where

V_R^0 = the retention volume corrected to the column temperature and average column pressure,

V_{gas} and $V_{11q.}$ = the volumetric gas and liquid hold-up of the column respectively at the temperature T.

The requirements for the accurate evaluation of γ^0 are:

(i) A very small sample size, or extrapolation of V_R^0 to zero size from a range of small samples.

(ii) Accurate measurement of retention time, dead-volume of apparatus, temperature and pressure of flowmeter, inlet and outlet pressures at the column, and column temperature.

(iii) Relatively long columns (3–6 m recommended [353] to reduce the effects of non-ideality in the first part of the column).

TABLE 3

VALUES OF γ_p^o FOR HALOGENATED HYDROCARBONS

Solute	*Solvent*						
	Silicone 702				*Dinonyl phthalate*		
	*20.2 °C**	*40.1 °C*	*77.0 °C*	*97.9 °C*	*39.7 °C*	*76.8 °C*	*97.9 °C*
CH_3Cl	0.36 (23.1)	—	0.37	—	0.45 (23.6)	0.45	—
CH_2Cl_2	0.38	0.40	0.44	0.50	0.42	0.46	0.49
$CHCl_3$	0.32	0.37	0.40	0.45	0.33	0.38	0.37
CCl_4	0.45	0.50	0.50	0.58	0.71	0.71	0.71
CH_2ClBr	0.42	0.45	0.47	0.51	—	0.49	0.48
CH_2Br_2	—	0.52	0.59	0.61	—	0.60	0.61
$CHBr_3$	—	—	0.65	—	—	—	0.64
CF_2Cl_2	0.75 (21.9)	0.76	0.63	—	0.91 (24.5)	0.80	—
$CFCl_3$	0.56 (21.9)	0.53	0.52	—	0.80 (24.5)	0.66	—
$CHFCl_2$	0.36 (23.1)	—	0.38	—	0.28 (23.6)	—	—
CHF_2Cl	0.76 (23.1)	—	0.71	—	0.58 (23.6)	—	—
cis-Dichloro-ethylene	—	—	0.42	0.51	0.38	0.42	0.42
trans-Dichloro-ethylene	—	—	0.46	0.53	0.52	0.58	0.60
Trichloro-ethylene	—	—	—	0.57	0.46	0.55	0.60

* Other temperatures are given in parentheses.

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(iv) Accurate determination of the mass of the solvent and the volume of the solvent at each value of T (which requires a knowledge of the coefficient of expansion), and correction or compensation for any loss of solvent due to its volatility.

(v) The molecular weight of the solvent (γ^0 values cannot therefore be accurately evaluated for solvents containing a mixture of substances in unknown proportions, or for polymers for which accurate molecular weights are not known).

Values of γ^0 which have been calculated by these methods are: aliphatic and aromatic hydrocarbon solutes in C_8–C_{10} hydrocarbons [373], C_{16}–C_{36} hydrocarbons [353, 364, 373], 1,2,4-trichlorobenzene [373], diisodecyl phthalate [353, 364], and polyalkylene glycols [353, 364]; oxygenated solutes in *n*-hexadecane (C_{16}) [373], diisodecyl phthalate [353], and poly-alkylene glycols [353]. Values of γ^0 for several halogenated hydrocarbons in dinonyl phthalate and in silicone oil 702 have been calculated [251] (Table 3) from the data of POLLARD AND HARDY [489].

The determination of the heat of solution

The apparent heat of solution (ΔH) and the excess partial heat of solution (ΔH_S^E) [492] can be derived from a plot of the logarithm of the partition coefficient (k) against the inverse of the absolute temperature:

$$\frac{\mathrm{d} \ln k}{\mathrm{d}\left(\frac{1}{T}\right)} = -\frac{\Delta H}{R} = -\left(\frac{\Delta H_v - RT - \Delta \overline{H}_S^E}{T}\right),$$

where

ΔH_v = the latent heat of vaporisation of the solute at its boiling point,
R = the gas constant.

Alternatively ΔH can be obtained by plotting $\log_{10} V_g$ against $1/T$ when [395]

$$\Delta H = -2.3R \text{ (gradient)} + RT^2 \, \mathrm{d} \log \varrho / \mathrm{d}T,$$

where

ϱ = the density of the solvent.

Straight line plots are usually obtained over the range 20–100 °C, but when the plots are slightly curved the value of the gradient is taken at the boiling point of the solute.

Heats of solution have been obtained for (i) several hydrocarbons and oxygenated compounds in the solvents squalane and diisodecyl phthalate [492], silicone 702 and tritolyl phosphate [395], (ii) for halogenated hydrocarbons in silicone 702 and dinonyl phthalate [489], (iii) for benzene and cyclohexane in liquid paraffin [14], and (iv) for several hydrocarbons in paraffin wax, liquid paraffin, and dimethyl-dioctadecyl-ammonium bentonite [593].

The determination of the entropy of solution

The entropy of solution (ΔS) of a solute in a solvent at temperature (T°A) can be calculated from the heat of solution (ΔH) and the free-energy of solution (ΔG) by the relation

$$\Delta S = \frac{\Delta H - \Delta G}{T}$$

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The value of ΔG has been calculated from the retention volume or partition coefficient at a given temperature by the equation [14]

$$\Delta G = -RT \ln k_B$$

in which k_B is Bunsen's partition coefficient defined by $k_B = 273k/T$.

White and Cowan [593] obtained entropies of solution analogously, but using the chemical potential of the solute ($\Delta\mu_n$) at a definite mole fraction, *e.g.* $n = 0.1\ M$, calculated from the vapour pressure of the solute exerted above a $0.1\ M$ solution under ideal conditions.

Values of the entropy of solution have been calculated from gas-chromatographic data for only a few systems: (i) for benzene — 13.2 e.u., and cyclohexane — 9.5 e.u. in polyethylene glycol cresyl ether [14], (ii) for homologous series in silicone 702 and in tritolyl phosphate [471]; in silicone 702 the addition of 3 carbon atoms along a series resulted in an average change of 1 kcal/mole in ΔG at 70° C, and 3 entropy units in ΔS; in tritolyl phosphate an average change of 2 kcal/mole in ΔG and 6 e.u. in ΔS respectively were found, and (iii) for benzene, toluene, *o*-xylene, hexane, cyclohexane, and heptane in liquid paraffin [593]; values of $-\Delta\mu_n$ and $-\Delta S_n$ from 1.44–1.80 kcal/mole and 15.1–16.9 cal/mole deg. respectively were obtained (n referring to a $0.1\ M$ fraction).

4. APPARATUS AND TECHNIQUES FOR G.L.C.

This section deals with apparatus for analytical and preparative gas–liquid chromatography and the techniques for quantitative analysis. For elution analysis one requires a supply of a suitable carrier-gas at a constant flowrate, a sample-introduction system, a column containing a stationary phase, and a detector.

(*a*) *Apparatus*

The mobile phase

The gases most frequently used for the mobile phase are nitrogen, hydrogen, helium, and carbon dioxide. High molecular weight gases lead to better separation in the gas–liquid column because of the reduced axial diffusion of the solutes, but the choice of mobile phase is also intimately connected with the detector. Hydrogen and helium have lower densities and higher thermal conductivities than nitrogen and both increase the sensitivity of detecting systems using these properties and also simplify the quantitative interpretation of chromatograms. However the efficiency of separation has been shown to be lower for hydrogen than for nitrogen [353, 357]. Carbon dioxide has been used with considerable success [113, 326–31, 353, 502] but the detection method (see later) limits the solutes which can be separated to those which do not condense at room temperature and which are not attacked by alkali.

The column efficiency depends upon the linear gas velocity which is not uniform along the column. There is an optimum velocity for a given set of operating conditions, but usually the gas flowrate is not a highly critical parameter. The volumetric gas

rate used depends upon the column diameter, among other factors, and flowrates for 6 mm diameter columns range from about 10–400 ml/min. A value of about 100 ml/min is usually satisfactory for this diameter of column, but for columns of greater diameter the flowrate should be increased in proportion to the square of the diameter, in order to maintain approximately the same linear rate.

The flowrate can be controlled with either a simple manometer [314, 323] or a well constructed needle valve, and measured by a number of methods, *e.g.* capillary, U-tube, and moving-bubble flowmeters, and rotameters. The latter are useful for indicating the flowrate but are not recommended [353, 580] for accurate measurements in the calculation of retention volumes.

The required flowrate is obtained by applying a pressure difference between the column inlet and outlet. Frequently either the inlet or outlet are at atmospheric pressure, but sometimes both pressures are controlled. Very long columns can be used for difficult separations without too excessive a pressure drop across the column provided that they are packed with a coarse supporting material (30–60 BSS mesh). Operation of columns at reduced pressure does not appear to offer any very great advantages [353], and usually leads to less efficient separation. Some gain in detector sensitivity is to be expected from operation of certain detectors at low outlet pressure but thermal conductivity detectors in particular become more pressure-sensitive at low pressure.

The sample and sample-introduction systems

The sample to be analysed may be introduced into the apparatus as a gas, a liquid, or a solid dissolved in a suitable solvent. Volumes introduced can vary from 0.1 ml to 1 μl for liquids and up to 20 ml N.T.P. for gases on a 4–6 mm diameter analytical column. Introduction of liquids with a micrometer syringe and hypodermic needle through a self-sealing rubber cap has become a standard technique, and many improvements of Ray's original method [501–2] have been suggested [91, 119, 571]. The introduction of reproducible amounts of liquids in glass capillaries through large bore metal valves is used in some recent commercial equipment [566–7]. For good resolution the whole of the sample should be introduced as rapidly as possible. This is best achieved by injection of the sample directly on to the column packing or on a glass-wool plug maintained at the column temperature. Alternatively a separate heater can be used at the inlet point to provide rapid vaporization [63, 187, 250, 486, 566] and the effect of the temperature of the heater on the column efficiency and retention volume has been studied [250, 254, 486]. Special methods for the introduction of samples from biological materials are required, *e.g.* liberation of free fatty acids and bases from their salts [54, 314, 418].

Gas samples can be injected directly with a hypodermic syringe [502] but by-pass gas pipettes or mercury burettes are more generally used [248, 256, 353, 568]. By-pass systems are particularly useful for periodic analysis of gas-streams [26, 276, 616] and can be adapted to automatic control. Greaseless systems are of particular importance since the majority of gases and vapours are soluble in greases and can cause severe

cross-contamination, especially if trace components are to be analysed. Special lubricants for taps have been used [113, 238] to overcome this problem. Many techniques used for the introduction of samples into mass spectrometers or vacuum systems can be applied to gas chromatography systems [6, 133, 433, 521].

The chromatographic column

Various materials have been used to contain the stationary phase, *e.g.* glass, copper, polythene, and stainless steel, and in many different shapes, *e.g.* straight, coiled, and U-shaped. Typical analytical columns are of the order of 4–6 mm internal diameter and 3–10 foot long. For longer columns, coiling can lead to a considerable reduction in the size of thermostat and the slight loss of efficiency can be tolerated. Columns of larger diameter have been used for the routine separation of larger samples, *e.g.* 3 cm diameter for up to 10 g samples [186, 361].

The column is usually maintained at a constant temperature by a vapour-jacket or a thermostatted oil- or air-bath. Accurate control of temperature is necessary for good reproducibility of retention volumes and accurate quantitative analysis. Vapour jackets are very convenient but the temperature is dependent upon the atmospheric pressure and for long term constancy a thermostat is better. In order to obtain a range of temperature with only one vapour jacket, the pressure above the liquid can be controlled with a manostat, but variable temperature air-baths are becoming widely used [23, 353].

Details have been given of columns operated under "programmed" heating, *i.e.* with the temperature increasing with time [124, 171, 231, 240–1, 246, 255, 523]. This method has advantages for the rapid separation of mixtures of substances having a wide range of boiling points, but a major difficulty is the maintenance of a constant base-line in the detector. Multiple columns in series with take-off points for the slower moving substances have also been used to decrease the analysis time [70, 103, 134, 201, 238, 255, 391, 432, 455, 538, 568, 617].

The stationary phase

(*i*) *The solid support.* Carefully prepared kieselguhr (Celite) has been used by many workers, and details of its preparation and purification have been given [314, 471]. More recently, a coarser form of kieselguhr, ground furnace-brick, has been recommended and the optimum particle size found to be 30–80 BSS mesh [141, 353, 357]. The advantages of the latter are that it is free-flowing and easy to pack into columns, and it has a low pressure-resistance so that long columns can be used without an excessive pressure-drop. The kieselguhr is often not completely inert and its weak adsorption leads to elution peaks with slight tails. This has been partly prevented for fatty acids by treatment with a solution of dilute phosphoric acid [314] or for bases by treatment with methanolic sodium hydroxide [319]. The treatment may saturate a small number of very active sites in a similar way to the reduction of tailing on active charcoal by the addition of a small amount of an involatile liquid, *e.g.* squalane [180–1], or the de-activation of clay by treatment with glycerin in adsorption chromatography

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[128]. The latter experiments with glycerinated clay may have been the first G.L.C. separations, although it was not realized at the time.

The method of mixing the liquid phase and the support can influence the separation efficiency of the column and the recommended method is to dissolve the liquid phase in a volatile solvent, to slurry the mixture with the support, and to evaporate the solvent with continual stirring.

Many other types of support have been used with varying success. Silicone rubber vulcanized and ground to a suitable size has been used for the separation of fluorocarbons [186] and can be used either alone as a dry column packing, or mixed with a conventional liquid phase. Glass powder has been used successfully by several workers [123, 394] but samples of some types of glass can give very inefficient separations compared to kieselguhr [488] and the resultant glass–liquid mixture is often difficult to pack. It was concluded [488] that for high-efficiency columns a porous support is preferable to a non-porous one. Stainless steel [545] or copper [373] helices give a very low pressure drop [545] and negligible interaction even with highly polar solutes [373], but the efficiency is low. Sodium chloride crystals have been used [118–9] at high temperature in preference to Celite in order to obtain a lower resistance to flow. Zeolites have been used as supports for liquid phases [326] in addition to their use as a solid adsorbent [31, 326, 592–4, 597]. Another, and perhaps novel, support reported is carborundum [557].

Capillary tubes coated internally with an involatile liquid (*without a porous packing*) have recently been shown to give good resolution and rapid separations, but require a very small sample and a very sensitive detector [158, 228–9]. The potentialities of this development are very great and in the future very high efficiencies are to be expected, *e.g.* a 250-ft. 0.010 in. internal diameter copper capillary coated with approximately 60 mg of squalane separated a 2 μg sample of 9-isomeric C7-paraffins in 50 minutes with an efficiency of 106,000 theoretical plates [143].

(*ii*) *The liquid component of the column packing.* Ideal requirements for the liquid phase are that it should be relatively involatile and stable at the temperature of operation, it should have a low molecular weight and low viscosity, and that it should be readily available in high purity. As many of these requirements are directly opposed to each other, some compromise is effected in practice. Although the selection of a suitable liquid phase for a given separation has often been empirical, considerable attention is now being given to this problem (see p. 7).

The following liquid phases have been recommended [580] for the intercomparison of solutes and for the presentation of retention volumes of new substances (useful temperature range given in brackets):

n-hexadecane (20–50° C)	dinonyl phthalate (20–100° C)
squalane (20–150° C)	dimethylformamide (— 20– + 20° C)
benzyldiphenyl (80–150° C)	diglycerol (20–100° C)

As a standard, a mixture of 20 parts by weight of liquid phase to 80 parts by weight of Celite 545 support should be used [580]. The above liquids are now being prepared in high purity commercially for special use in gas chromatography.

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When liquid phases are operated at high temperature, *e.g.* > 100° C for many liquids, a number of problems are encountered including the loss of liquid phase by vaporization and by decomposition [3, 23, 90, 93, 119, 190, 246, 270, 351, 566–7]. The development of stable liquid phases of low volatility is of great importance because of the opportunity of extending gas chromatography to the separation of organic and inorganic materials of low volatility. A number of stationary phases have already been used successfully for long periods at up to 300° C and these include silicone greases, apiezon greases, silicone elastomers, silicone-gum, and polythene. Many other materials have been examined and found to be less satisfactory; for examples, refer to those rejected by KEPPLER and co-workers [351].

The detector

A large number of types of vapour detector have been used for the detection and recording of the separations achieved by the chromatographic column. The detectors can be divided into two main divisions, (A) integral detectors and (B) differential detectors. An integral detector measures some function of the total quantity of vapour which has passed through it, while a differential detector measures some function of the vapour concentration. The latter can in principle be converted into an integral detector by an electronic or mechanical integrator.

The ideal requirements for a detector are: high sensitivity to the presence of a component in the carrier-gas; rapid response; linear response; independence of operating variables such as pressure and flowrate of gas; good base-line stability; simplicity of construction and auxiliary equipment; robustness; low-cost. BOER [53] compared eight types of differential detector on the basis of many of the above requirements and concluded that thermal conductivity detection and β-ray ionization detection appeared to be, at that time, the two systems most suitable for a wide range of applications in routine laboratories. However, the detectors in widespread use are (a) thermal conductivity, (b) gas-density balance, (c) hydrogen flame, and (d) direct measurement of gas-volume after absorption of the carrier-gas. Methods not considered by BOER, or which have appeared since then, are based on interferometry, measurement of dielectric constant, velocity of sound, or ionization potential at low pressure.

A. Integral detectors

(*a*) *Measurement of volume or pressure.* JANAK and co-workers [330] used carbon dioxide as the mobile-gas to carry the separated components of mixtures from the column into a nitrometer containing a concentrated solution of potassium hydroxide. The carbon dioxide was absorbed and the residual gases measured in a burette. The method is limited to gases and vapours which do not condense at room temperature and which are not attacked by alkali. Much valuable work has been done with this simple type of apparatus and automatic recording models have recently been developed [327, 377, 535]. VAN DE CRAATS [113] also described recording integral detectors based on the measurement of the volume, or of the pressure in a constant volume

chamber. A convenient method of purification of CO_2 for this detector is given by KEULEMANS [353].

(*b*) *Direct titration.* JAMES AND MARTIN [313–4] directly titrated volatile fatty acids and bases by passing the gas stream from the column through a titration cell, and gave details of automatic and manually operated micro-burettes. A number of modifications of the original methods have since been described [418]. BLOM [50, 51] has described a simple and inexpensive method based on combustion of the vapours over copper oxide. The CO_2 formed is titrated in a glass apparatus with pyridine as solvent and sodium methylate in pyridine–methanol as titrant. This has been applied to 1–10 mg mixtures of a wide variety of organic substances including nitrogen-, sulphur- and halogen-derivatives.

(*c*) *Other methods.* Mercaptans have been estimated [557] by iodine oxidation in a redox half-cell by passing the vapours through the column with nitrogen and absorbing them in a solution of iodine in potassium iodide/ 70% alcohol which formed a redox half-element. In a 100 μg mixture each component could be quantitatively determined to 0.03–0.04 μmole. With a small excess of iodine over mercaptan the sensitivity was as high as 10^{-9} mole/mV output. However, tertiary mercaptans cannot be oxidized by this method.

For the analysis of fatty acids BOER has described [54, 55] a coulometric titrator coupled to a recorder, and also an electrolytic conductivity cell. The latter is simple and only requires a thermostatted titration vessel containing dilute sodium hydroxide, a conductivity cell, and a slightly modified electronic recorder. It can be adapted to other detecting systems such as bases with sulphuric acids, or ketones with hydroxylamine chloro-hydrate [44, 54]. LIBERTI has also described [381–6] two methods involving coulometric titration, one for components with an active chemical group, *e.g.* acid or base, and the other for general application.

In the first method the vapours are absorbed in a cell containing a suitable indicator and are titrated with a generating electrode connected to a coulometer in which the current can be controlled by a photo-electric relay.

In the second method oxygen is used as carrier-gas and a small furnace at the exit of the column burns each component to CO_2. The oxygen and CO_2 bubble through barium chloride solution in a cell containing a Pt-wire which acts as an oxygen-electrode and shows the presence of each component. Coulometrically generated hydroxyl-ions bring back the potential to its initial value.

JANAK and co-workers [331] have determined halogens polarographically by eluting them from a silica-gel column and following the increase in the Ti(IV) wave after absorption of the halogens in a Ti(III) solution. NEDOROST [449] has given details of a polarographic cell for continuous gas analysis.

B. Differential detectors

(*a*) *Thermal conductivity.* The measurement of thermal conductivity has been used for many years for the analysis of simple mixtures of gases, and details of the general design and operation of thermal conductivity cells (or katharometers) are given by

DAYNES [138], WEAVER [585], and MINTER AND BURDY [434]. The principle of the detector is that if a constant flow of gas passes over a fine wire heated by a constant electric current the rate of loss of heat by the wire is constant. A change in composition of the gas stream will cause a change in heat loss and thus a change in resistance. In practice two wires and two streams are generally used, one stream from the chromatographic column and one from a source of gas of constant composition. The wires are connected in a Wheatstone bridge circuit and any change of bridge output, due to a change of wire resistance, is amplified and recorded.

Thermal conductivity cells have found widespread use in gas chromatography because of their simplicity, low cost, general applicability and comparatively high sensitivity. They were used in 1936 by EUCKEN AND KNICK [471] to detect gases removed from a column of adsorbent and were also used by many the of early workers in G.S.C. and G.L.C.

A variety of metal-block, and glass, cells with straight and coiled platinum and tungsten wires have been described [8, 57, 59, 60, 63, 75, 101, 117–8, 130, 138, 159, 178, 184, 190, 194, 213, 236, 241, 257, 309, 351, 358, 390, 402, 429, 430, 434, 459, 477, 483, 501, 516, 522, 525, 543, 555, 567, 596, 608]. Optimum operating conditions and sensitivity have been studied by many workers and have recently been discussed in detail by KEULEMANS [353].

The general conclusions from studies of the sensitivity and stability of cells operated with various carrier-gases are that a higher sensitivity can be obtained with hydrogen and helium than with nitrogen, but in most cases this leads to lower stability. The use of hydrogen or helium instead of nitrogen can have definite advantages for quantitative analysis since the thermal conductivity of volatile materials is then less dependent upon the type of molecule and the amount of calibration can be reduced. The sensitivity can be increased for small amounts of impurities by combustion of the vapours to (i) CO_2 [236, 272, 450], (ii) methane [618–9], or (iii) hydrogen [236] which are subsequently detected. The behaviour of the thermal conductivity cell depends considerably upon the temperature of the wire and of the walls of the cell. Incorrect choice of wire temperature can lead to loss of sensitivity and even complete insensitivity to particular substances, *e.g.* non-polar molecules [257]. The inclusion of the katharometer in the column heating chamber is not recommended if maximum sensitivity and resolution are desired. Independent heating is advisable if it is necessary to avoid condensation in the cell.

Thermal conductivity cells for continuous operation at high temperature, *e.g.* up to 350° C, have been developed [23, 38, 117–9, 127, 130, 190, 194, 351, 566–7] and some design implications of high temperature operation have been discussed [130, 351]. The problems are largely those of choosing suitable materials to withstand temperatures of 300° C and upwards, particularly for electrical insulation and gas seals. A comparison of copper, stainless steel, and glass cells [351] showed that metal cells could be used for temperatures up to 250° C, and glass cells up to 300° C. With a 0.67 mm diameter glass cell and a 20 μ diameter wire operated at 8 V, a minimum amount of 0.5 μg of methyl caproate could be detected as a peak eluted from a column

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in nitrogen. A compact and simple detector (up to 500° C) has been described using "glow plugs" as elements in a diffusion type cell [192–4].

Thermistors are becoming more widely used in place of metal wires and advantages claimed are low dead-volume, and short response time [27, 40, 78, 124, 131–2, 258, 277, 346, 455, 459, 564–8, 570]. Satisfactory thermistor detectors are now operating in many laboratories and more detailed work is available on stability, sensitivity and optimum operating conditions [40, 112, 583]. DAVIS AND HOWARD [131, 132] described a detector which was stable for long periods and had a low noise level provided that the flowrate of gas, and the temperature of column and detector, were kept constant, and that the whole system was allowed to reach thermal equilibrium before samples were eluted. They found that the magnitude and the direction of the signal varied with the distance of the detector from the exit of the column, and proposed that the heat capacity of the gas was measured by a detector in close proximity to the column exit, whereas the thermal conductivity of the gas was measured by a detector at a distance from the column exit. The development of thermistors to work at high temperature with high stability and sensitivity may be difficult due to the resistance–temperature curve falling sharply with increasing temperature. BISHOP [48] has compared a number of commercial thermistors and has shown that the curve relating sensitivity and operating current passes through a maximum (see also ref. [112]). When operated in this region the thermistor detector has a comparable sensitivity to the "wire" thermal conductivity cell. Care must be taken that the thermistor element is not reduced at high temperatures when hydrogen is used as carrier-gas.

(*b*) *Gas-density balance.* CLAESSON [101] in 1946 measured the density of the gas from a chromatographic column by means of a liquid manometer connected to two 6′-long columns of gas, one containing the effluent gas and the other a standard gas.

MARTIN AND JAMES later developed an instrument based on the same principle but having extremely high sensitivity [411]. The instrument, called a gas-density balance, consists of a metal block bored with a series of tubes which are connected together in a manner analogous to a Wheatstone bridge to compensate pressure differences due to the flow of gas. The pressure difference set up between two of the tubes is then a function of the gas-density only, and the density of the effluent gas from the chromatographic column is compared to the density of the carrier-gas from a "dummy" column. Any difference in density causes a flow of gas in a cross-channel in the metal block. This channel contains a flow-detector consisting of an electrically heated filament close to two connected thermojunctions and a flow of gas alters the temperature of these thermojunctions. The thermoelectric output is amplified and recorded, and for small density differences the recorder deflection is linearly related to the density difference of the two gas streams.

MUNDAY AND PRIMAVESI [444] have constructed an electrical analogue of the system to evaluate the performance, and have compared a copper-block gas-density balance with a balance constructed from copper tubing of the same internal dimensions as the tubes in the solid block. The response curves of the two models were very

similar, the solid block model having slightly greater sensitivity and lower background noise. The balances gave a response which was linear with the density difference over the range of practical importance, and the authors discussed the reasons for non-linearity at higher density differences. HAWKES has given details [270] of the construction of a gas-density balance for operation at temperatures up to 300° C, but sensitivity decreases as the temperature is raised, *e.g.* the sensitivity at 56° C is 20% less than at 20° C. An instrument constructed of Monel tubing has been described [184] for the analysis of corrosive and highly reactive gases. Many other applications have been reported [38, 149, 234, 296, 302, 309] and the gas-density balance has been used to determine the molecular weight of solutes to ± 4% [388].

(*c*) *Hydrogen flame detector.* This detector was introduced in 1955 by SCOTT [526] and has since been studied in detail by a number of workers [127, 183, 279, 527, 605]. Hydrogen or a mixture containing hydrogen is generally used as the carrier-gas and is burnt at a small jet at the exit of the column. The temperature of the flame is measured by a thermocouple (for flame temperature contours see PRIMAVESI [495]) and the area of a peak on the recorded chromatogram, corrected for the heat of combustion of the substance, is proportional to the weight of substance present. The detector has the advantages of simplicity, low dead-volume, and low cost, and can be applied to gas chromatography at high temperature. SCOTT used hydrogen and nitrogen–hydrogen mixtures as carrier-gases and found a linear relationship between peak height and weight of substance present, but the sensitivity decreased as the retention volume increased. A single straight-line plot was obtained for peak area against sample weight for a number of hydrocarbons. HARRISON [254] found that pure hydrogen is not a suitable carrier-gas for the elution of methane but that 75:25 or 70:30 hydrogen–nitrogen mixtures are satisfactory. WIRTH [605] has reported the accuracy and reproducibility of quantitative results and compared the response of oxygen, water, and oxygenated and chlorinated compounds with the response of hydrocarbons. He used nitrogen carrier-gas and mixed it with hydrogen (from a dummy column) at the column exit. HENDERSON AND KNOX [279] found that the temperature rise of the thermocouple during the combustion of any band is directly proportional to the rate of liberation of heat, and obtained a linear relationship between the peak area per mole and the molar heat of combustion for twenty-four substances.

GRANT AND VAUGHAN have described [233, 235] a micro-flame, or emissivity, detector which measures the luminosity of the flame rather than its temperature. The hydrogen gas from the column is carburetted with benzene or mixed with coal-gas before burning in the detector. The detector is at least as sensitive as the katharometer and possesses several advantages. It is of simple construction and is particularly suitable for use at high temperatures. Also the response depends largely on the nature of the eluted solutes, hence by comparison of the responses of the emissivity detector with those of a katharometer, a classification scheme can be evolved.

MCWILLIAM AND DEWAR have recently described [427–8] an ionization detector using a hydrogen-flame. This is claimed to have extremely high sensitivity (*e.g.*

$S = 1 \cdot 10^9$ mV.ml/mg [53, 160] with a background of 0.1 mV and compares favourably with other types of ionization detector (see later). In its simplest form, a small platinum loop or brass gauze forms the negative electrode and is supported from 5–10 mm above the jet from which the nitrogen–hydrogen mixture is burnt. A potential of from 0–200 V can be applied between the earthed jet and the negative electrode and the output taken across a high resistor between the jet and earth. A similar instrument has been described by HARLEY *et al.* [252].

(*d*) *Ionization detectors.* The measurement of the ionization current in a gas has been used for analytical purposes by a number of workers. A method based on ionization by β-rays from a radioactive source was developed for gas analysis [491] and later applied to detection in gas chromatography [53, 139]. The advantages of this system are that it is very sensitive; calibration is virtually unnecessary since the differential ionization current may be predicted from the ionization cross-sections of the component molecules; the cell is simple to construct and is adaptable to high temperature; and it is inherently insensitive to changes in flowrate of carrier-gas (N_2 or H_2). Disadvantages are that the auxiliary equipment is more elaborate and costly than, for example, the thermal conductivity method, and precautions are necessary in handling the radioactive source. A sensitive and stable detector has been described using argon as the carrier-gas and a β-radioactive source [398], but to reduce or prevent radioactive handling problems, a number of other methods have been considered. The simplest and most effective is that based on a spark discharge operating at atmospheric pressure [399].

HARLEY AND PRETORIUS [253] introduced a detector in which the potential difference was measured across two electrodes in a high-voltage (900 V) discharge tube through which a part of the carrier-gas passed at a few mm pressure. Less than 10^{-11} mole of a hydrocarbon could be detected and a similar figure has been given by PITKETHLY [479, 480] who recommends modified small neon indicator lamps, or aged iron electrodes from neon lamps.

RYCE AND BRYCE [522, 524] have developed a sensitive low-voltage ionization detector. A small fraction of the helium gas stream from the column passes through a leak into a modified commercial ionization gauge operated at $\sim$ 18 V which is not sufficient to produce ions when helium alone is flowing through. Solutes from the column have ionization potentials below 18 V and give rise to a plate current which is amplified and recorded. The sensitivity claimed is 200 times that of the thermal conductivity method and compares with that of the high-voltage detector. The detector is insensitive to changes in ambient temperature, pressure, and flowrate of the main gas stream of which only a small fraction ($< 0.5\%$) is used.

(*e*) *Radioactivity detectors.* GLUECKAUF, BARKER AND KITT [220] followed the separation of gases by measurement of radioactivity and this is the most sensitive method if the materials to be detected are radioactive. EVANS AND WILLARD [188] have recently demonstrated the detection of amounts of the order of 10^{-15} g of $CH_3{}^{80}Br$ or 10^{-13}g of $CH_3{}^{82}Br$, the sensitivity being inversely proportional to the half-life of the respective radioactive isotope, ^{80}Br (18 min), ^{82}Br (36 h). These authors

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used a Geiger counter, or a sodium iodide scintillation counter coupled to a photomultiplier, amplifier, ratemeter, and recorder, and separated substances from columns operated at up to 200° C. These results indicate that gas chromatography is as effective with materials at "trace"-levels as at macro-levels. KOKES and co-workers [369] also describe the use of a Geiger counter to detect radioactive components separated from catalytic reactor mixtures. Apparatus for the detection of tritium and ^{14}C-labelled compounds by their weak β-radioactivity has been described [4, 79, 231, 400, 441, 507, 610].

(*f*) *Surface potential detector.* GRIFFITHS, JAMES AND PHILLIPS [241–2] introduced a detector based on the measurement of the e.m.f. set up over a vibrating condenser formed by two metal plates, one of which is coated with a suitable surface film (*e.g.* stearic acid). The detector gives a high sensitivity with polar vapours and the sensitivity increases with molecular weight, but it has a slow and non-linear response. However, it has been successfylly applied to displacement analysis in G.S.C.

(*g*) *Other detectors.* MARTIN AND SMART [404], LIBERTI and co-workers [389], and others [200, 272, 531] have applied infra-red analysers to gas chromatography. Organic vapours are passed in the carrier-gas over heated copper oxide and converted to carbon dioxide. With an infra-red analyser sensitive to carbon dioxide a high sensitivity (proportional to the number of carbon atoms in the molecule) is obtained, provided that combustion is complete. The method is then limited to carbon compounds and a considerable disadvantage is the destruction of the chromatographed material. MARTIN AND SMART suggested that for infra-red absorbing vapours a detector sensitive to all the vapours present would suffice but then the sensitivity would not be high, and would vary with the vapour. However, LIBERTI [389] has reported that hydrocarbons can be analysed satisfactorily without combustion by measuring the absorption at the C-H band frequency, 3330 cm^{-1}.

GRIFFITHS and co-workers [241] investigated methods of detection based upon the measurement of (i) the specific heat of the effluent-gas, (ii) the latent heat of adsorption of vapours, (iii) changes in the dielectric constant of a section of the column as the vapours passed through, and (iv) the flow impedance of the carrier-gas. Only (ii) and (iii) have received any recent application. A simple and robust detector of changes in dielectric constant has been designed [578] for use with routine preparative columns, but has high enough sensitivity for many analytical applications.

DUDENBOSTEL AND PRIESTLEY have reported [173] a detector based on the measurement, by thermocouples, of the differential heats of adsorption and vaporization as the carrier-gas passes through a small bed of an adsorbent at the end of the chromatographic column. The detector is sensitive to low concentrations of hydrocarbons in air and was applied in a continuous process-plant to the analysis of propane in propylene. PRIESTLEY reported [580, p. 165] an improvement whereby a thermocouple inserted directly into the end of the column gives a signal which is proportional to the amount of a component passing the thermocouple.

The measurement of sound velocity has been suggested [310] as a method of detection but the present difficulty is the large volume of sample required to give a

sensitive and reliable response. Gas interferometers have been used [577, 614] in gas–solid chromatography but such instruments have never come into general use.

Additional apparatus

The collection of fractions is important not only for the application, if necessary, of further methods of analysis to resolve complex mixtures, but also for the isolation of substances in high purity, and for the measurement of their physical properties. Details of apparatus and methods are given under preparative gas chromatography. The measurement of physical properties can easily be achieved on very small amounts of substances, *e.g.* by mass-spectrometry, but larger amounts are usually required for infra-red [39, 165, 207, 447] or ultra-violet spectrometry [401]. However, micro-cells requiring only 0.002 ml of liquid are now available with commercial infra-red spectrometers. James and Phillips [324] have described a simple micro-Schliermacher apparatus for the determination of boiling points to 0.1° C on 0.02 ml of liquid. Littlewood [393] has developed an effusiometer for the determination of molecular weights of boron and silicon hydrides to 5% on samples of 10–100 μg. Liberti and co-workers [388] have measured molecular weights with an accuracy of about 4% by eluting the mixture of an unknown and known substance in two different carrier-gases of very different molecular weight. From the ratios of peak areas in each gas the molecular weight of the unknown can be calculated.

The combination of mass spectrometry and gas chromatography can be of great value. Bradford and co-workers [63] described in 1955 an analysis in which a complex petroleum mixture gave sixteen chromatographic peaks with a thermal conductivity detector, each of which was collected and analysed by a mass spectrometer. Thirty-two components were finally identified in the mixture. Drew and co-workers [172, 422–6, 542] applied mass-spectrometry to the positive identification of chromatographic peaks and the analysis of mixtures having identical emergence times (particularly deuterated hydrocarbons). They also used gas chromatography to separate mixtures of hydrocarbons which have almost identical cracking-patterns so that mass-spectrometric analysis alone had not previously been possible. The combination of gas chromatography and mass-spectrometry has been reported by a number of workers [45–6, 80, 137, 169, 172, 206, 222, 224, 230, 286, 348, 380, 510, 532, 550, 551].

Commercially available apparatus

In the last few years a number of gas chromatography apparatus have become available commercially. At the present time a variety of types are marketed by a large number of manufacturers in the U.S.A. and Great Britain. The apparatus is robust and the reproducibility of quantitative analysis and sensitivity in qualitative analysis of trace materials is usually of a high order. Prices in Great Britain are of the order of £ 600–£ 1000. The most recent development is the application of gas chromatography to continuous automatic analysis of, for example, liquid and gaseous

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process streams in industry [5, 26–7, 77, 173–4, 176, 191, 198–9, 271, 276–8, 368, 461, 483, 549–51, 591, 616].

(*b*) *Techniques*

Quantitative analysis

A quantitative analysis can be obtained from measurements on the recorded chromatograms, the method depending upon the type of detector used and the nature of its response to the components passing through in the carrier-gas. In quantitative analysis with integral detectors the step heights recorded in the chromatogram directly measure the amount of substance separated, for example, in terms of volume, or pressure, of a gas, or μg equivalents of acid or base etc.

With differential detectors in which the chromatogram is a series of peaks on a chart the quantity of a substance can be related to the height of a peak or the area under a peak. The area can be measured by a mechanical or electronic integrator [125, 353, 554], a planimeter [177, 390, 489], cutting out and weighing the chart [159, 516], or by multiplying peak heights by half-widths [116]. With a small sample, the peak height is proportional to the quantity of a component [501–2] and this affords a very simple and rapid method of quantitative analysis. For detectors which have a response dependent upon the nature of the substance in the carrier-gas, calibration for each substance is usually necessary. With thermal conductivity detectors using nitrogen as carrier-gas, calibration is necessary, but with hydrogen or helium carrier-gas (which have thermal conductivities higher and very different from those of the vapours) the response is less dependent upon the type of vapour molecule. However, for the most accurate results, calibration for each substance is recommended [78, 114, 160].

In gas chromatography it is difficult to introduce quantitatively small samples of liquids and gases and to keep the operating conditions constant. These problems can be largely overcome by using either an internal standard or internal normalization.

In the *internal standard method*, a known amount of a suitable substance is added to the sample to be analysed and the peak-areas or -heights are referred to the area or height of the internal standard peak [63, 117–9, 501]. Dimbat and co-workers [160] investigated the effect of operating variables using a reproducible by-pass sample injection system and found that the height of recorded peaks is sensitive to column temperature but not to flowrate, whereas the peak area is sensitive to flowrate but not to column temperature. They consider that control of column temperature to at least 1° C and flowrate to $\pm$ 1% is required for quantitative analysis using peak areas. Ray [501–2] found that 1° C change in column temperature gave 2.4% error in determination of methylcyclohexane by the height relative to *n*-pentane. A 1° C change in column temperature gave only a 0.3% error for the above materials when peak areas were used [160]. The introduction of liquid samples under constant conditions is stressed by Pollard and Hardy [486]. They showed that the rate of change of peak height of an internal standard with temperature of injection may be significantly different from the rate of change of height of the peaks being analysed.

In the *internal normalization method* the areas of all the peaks present are added

to give a total area which is normalized to 100%. The ratios of the individual areas to this total give the weight percentage amounts directly. Calibration, or correction, factors can be applied in this and the previous method when mixtures to be analysed contain substances whose responses to the detector are not independent of the type of substance, *e.g.* correction for heats of combustion with the hydrogen-flame detector [527], and for thermal conductivities [78, 358, 430, 516, 568].

The accuracy and reproducibility of quantitative analysis varies with many factors such as the type of detector, the method of analysis used, and the control of operating conditions. The simple detectors based on volume measurement or titration are claimed to be highly accurate (± 0.1% for the JANAK technique) but the sensitivity of these methods is somewhat limited, *e.g.* by the difficulty of accurate measurement of very small volumes of gas. The other detectors and methods, while more versatile and sensitive, can seldom be claimed to give results to better than ± 1% of each component, and it may not be possible to achieve this for the analysis of trace-components. However, it may sometimes be desirable to sacrifice some accuracy or reproducibility for speed of analysis. The reproducibility of particular methods of analysis has occasionally been quoted in terms of the standard deviation. DIMBAT and co-workers [160] reported standard deviations of 7–10% for methods using peak areas and a thermal conductivity detector without calibration, and 0.76% with prior calibration. POLLARD AND HARDY [489] give an average standard deviation of 1.4% for three determinations of halogenated hydrocarbons using peak heights relative to an internal standard. The difference between the amount found and the amount taken was usually under 2% for this method. PERCIVAL [465] analysed fluorinated hydrocarbons and gave 95% confidence limits for the mean of duplicates of about ± 0.5% at the 50% level for any component. The accuracy was within the reproducibility limits. LICHTENFELS *et al.* [390] report an average error of 0.6 mole % for the analysis of an 18-component mixture of hydrocarbons by peak areas, but all the peaks were not completely resolved.

Preparative applications of G.L.C.

Gas–liquid chromatography is primarily a batchwise method of separation of mixtures and has been developed more extensively as an analytical technique than as a technique for the preparation of pure substances. Analytical scale columns can be used to isolate constituents of complex mixtures in small amounts suitable for further study, *e.g.* by mass-spectrometry, but for very complex mixtures or small amounts of impurities, larger samples are desirable.

The amount of sample which can be eluted through a gas–liquid column can be increased in proportion to the cross-sectional area of the column without seriously impairing the efficiency of separation. EVANS AND TATLOW [186–7] separated g amounts of mixtures of fluorinated hydrocarbons on columns 16 ft. long by 3 cm diameter and more recently 16 ft. by 7.5 cm diameter [185]. The latter columns are capable of routinely dealing with 10–70 g of volatile mixtures and give separations previously unobtainable by fractional distillation because of azeotrope formation and

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the close similarity of boiling points. Compounds boiling 1° C apart, 3H- and 4H-nonafluoro-cyclohexene, were separated from each other. WHITHAM [595] has applied medium-scale, 830 cm (27 ft.) × 13 mm diameter, columns to the analysis of up to 3 ml liquid samples of complex petroleum fractions. BRADFORD, HARVEY AND CHALKLEY [63, 256] in 1955 also used comparable diameter columns for separating 0.5 ml samples of liquid hydrocarbons. They overcame any decrease in separation efficiency (due to larger sample size) by eluting at a lower column temperature. ROBB AND VOFSI [511] separated 500 mg quantities of mixtures of vinyl acetate and bromotrichloromethane on 140 cm (4 ft. 8 in.) × 10 mm diameter columns, and obtained quantitative recoveries to 1.5 mg. Several other applications have been described [66, 71, 92, 361, 371, 551, 559, 579].

AMBROSE AND COLLERSON [9] developed an apparatus for preparative gas chromatography in which the cycle of batch operation was automatically repeated. As each substance emerged at a constant time after sample-injection a clock was used to control the cycles, and solenoid valves directed the samples to collecting traps. The authors used 100 cm × 15 mm diameter columns and sample charges of slightly less than 1 g, but the method is applicable to any size of column. The success of the method depends upon the chromatogram remaining in phase with the clock. Constant elution times were achieved by control of column temperature, carrier-gas flowrate, and presaturation of carrier-gas with the stationary liquid phase. Developments of this type of apparatus have recently been described by a number of workers [25, 273, 287, 371].

The development of efficient collecting systems for samples eluted from gas chromatographic columns has received considerable attention, and a number of techniques have been discussed recently (see ref. [580]: *V.P.C. Symp.*, pp. 96, 209). Simple U-tube traps cooled in liquid nitrogen have been used with varying degrees of success. A relatively low pressure at the column outlet is claimed to facilitate quantitative condensation of components. Expendable traps have been developed with low hold-up volume and sintered glass packing for the recovery of very small fractions (less than 30 μg) from reaction kinetics studies [171].

The rapid cooling of mixtures of a carrier-gas and a condensable vapour often leads to loss due to the formation of fog or minute crystals. Without using filters a good recovery can be obtained by placing several cooling traps in series and heating the gas stream between the individual traps sufficiently to evaporate the floating droplets or crystals [580, p. 96]. GLUECKAUF [580, p. 97], finds that if traps are filled with cotton-wool or some other material on which droplets can collect, there is complete condensation up to the vapour pressure of the substance concerned. If the vapour pressure of the substance is too high at liquid nitrogen temperature, then a small plug of active charcoal can be used. Amounts of less than 10^{-12} g of krypton and xenon, were quantitatively retained by this method. AMBROSE [580, p. 210] reported that active charcoal retained organic materials too strongly and recommended activated alumina. An electrostatic precipitator is stated to overcome fog formation completely [25].

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Under certain conditions, small 4 mm diameter gas–liquid columns can be considerably overloaded in order to prepare small samples of pure substances, *i.e.* liquid samples of the order of 0.3 ml can be eluted instead of the normal 1–10 μl [487]. The required substance must have a sufficiently different retention volume from the impurities present. If the retention volumes of impurities and the major component are very similar, the column cannot be overloaded to the above extent, and a larger column is required. Pure ethyl nitrate and formic acid have been prepared by this method.

5. CONCLUSIONS

Gas chromatography has been used to separate complex mixtures of substances in amounts ranging from 10^{-15}–70 g and with boiling points from — 200° to + 400° C. It can both qualitatively and quantitatively analyse these substances with good accuracy and in the comparatively short time of a few minutes to a few hours. The separated substances can then be recovered in a high state of purity. Not only is it an outstanding technique on its own, but it can also be used in conjunction with methods such as fractional distillation, mass-, and infra-red-spectrometry, and can achieve results which were previously not possible with existing methods. The appearance of commercial instruments designed for application to research, to the routine analysis of all types of substances, and to process-control in industry, is greatly aiding in the adoption of gas chromatography as a universal separation-process.

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STARCH ELECTROPHORESIS

I. STARCH BLOCK ELECTROPHORESIS

H. BLOEMENDAL

Department of Biochemistry, Antoni van Leeuwenhoek-Huis: The Netherlands Cancer Institute, Amsterdam (The Netherlands)

1. INTRODUCTION

Starch is used as supporting medium in three zone electrophoretical techniques:

I. Starch block electrophoresis, introduced by KUNKEL AND SLATER[1,2].

II. Starch column electrophoresis, developed independently by CARLSON[3] and FLODIN AND PORATH[4].

III. Starch gel electrophoresis, first described by SMITHIES[5,6].

The name zone electrophoresis has been introduced by TISELIUS[7] in order to indicate a difference with moving boundary electrophoresis. While in the moving boundary or free electrophoresis one works in dilute solutions with the inevitable result of only partial separation of different fractions, in zone electrophoresis the application of a solid material as supporting medium allows the separation of a mixture into discrete zones.

Various materials have been used as stabilizing medium. Especially for the separation of proteins, peptides, and probably also ribonucleic acids, starch has some advantages over paper, gelatin, agar, silica, asbestos fiber, or glass powder.

(i) Adsorption of most proteins on starch is only small.

(ii) Elution of starch segments after the electrophoretic run is easier than in the case of gelatin or agar gel which is an important factor for preparative purposes.

(iii) Starch forms more easily a homogeneous paste with buffers than does cellulose or glass powder.

(iv) Electrosmosis is lower than in most other supporting media[2] although this phenomenon may cause poor separation also in the starch block technique, mainly when electrophoresis is carried out with horizontal arrangement of the starch block[8]. Starch electrophoresis is generally considered to be a rather gentle purification method for proteins, that is to say that there is no more chance of denaturation than there is in the method of salting-out or precipitation with acids, alcohol or acetone. SORKIN *et al.*[9] on the contrary claim that during the electrophoretic run irreversible denaturation processes lead to considerable loss of protein. The question arises whether this holds true for their special case or that heat development, caused by the 17–20 mA current in their experiments with 0.1 μ buffer solutions, was responsible for the observed denaturation. Buffers of lower ionic strength than 0.1 μ usually give better results.

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HARRIS AND MEHL[10] found a decrease of 40–50 per cent in enzymatic activity during electrophoresis of crude intestinal alkaline phosphatase. These authors could not give an explanation for this effect but were able to show that incomplete recovery from starch would cause a loss of 12–13 per cent only.

Hereafter we shall discuss the most important applications, and our own experience with starch block electrophoresis, which has been introduced as routine technique in the Laboratory of Anatomy and Embryology ((University of Amsterdam) and in the Department of Biochemistry of the Netherlands Cancer Institute.

2. METHODS

Apparatus

Starch block electrophoresis is carried out either in semicylindrical glass troughs or in rectangular plastic boxes which can easily be constructed in every laboratory. Glass troughs can be obtained by cutting Pyrex tubing in half longitudinally[11]. In our laboratory perspex boxes 40 × 2 × 4 or 80 × 2 × 8 cm appeared to be convenient for analytical and large scale preparative work. The use of a starch block simply packed in wax paper as originally described by KUNKEL AND SLATER is not advisable. When very high voltages are required a water-jacketed box may be used[12].

Electrode vessels

Reversible Ag–AgCl electrodes as well as agar or filter paper bridges between the electrode vessels are sometimes used[12–14, 56]. Very simple electrode vessels are made of

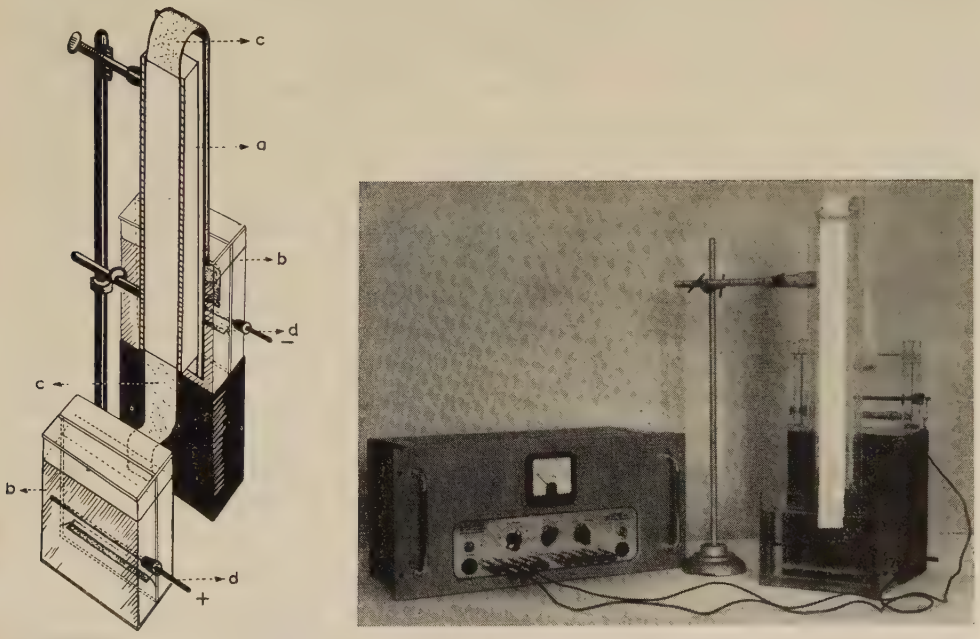

Fig. 1. Starch block electrophoresis apparatus [vertical arrangement of the block (a), perspex electrode vessels (b) with carbon electrodes (d)].

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perspex 18 × 7 × 14 cm. Each vessel is divided into two compartments by means of a perspex plate (Fig. 1, b). A cotton wick pressed into a slit in this plate serves as fluid bridge. Separated compartments are necessary to maintain the pH constant in that part of the vessel which is connected with the starch block. While there is a considerable change of pH in the outer electrode compartments, there is but a very slight decrease or increase, respectively, of pH in the inner compartments of the anode and cathode vessel. When experiments are carried out for longer than 24 hours it becomes necessary to renew the buffer solution in the electrode vessels. Inexpensive carbon rods (Fig. 1, d) appear to be satisfactory in various buffers, although at higher voltages carbon anodes show a tendency to disintegrate. In these cases they may be replaced by coiled platinum wire. Balance in the electrode vessels is obtained by connecting cathode and anode with a rubber tube. This tube has to be closed during the electrophoretic run. Balancing of the levels is unnecessary when the box is arranged vertically and the cathode vessel is placed higher than the anode vessel (Fig. 1). When a series of troughs at different pH's or ionic strengths are run at the same time, the anode chamber will have to be divided in subcompartments into which the troughs dip, as described by PAIGEN[12]. This arrangement protects the troughs from any change induced by electrosmosis. For the cathode vessel this division is not required.

During the electrophoretic run the vessels are closed with perspex plates or a sheet of parafilm.

Preparation of starch

Potato starch is the material most commonly used in starch electrophoresis. Depending on the purpose of a given investigation the starch has to be purified more or less carefully before use. If very exact analytical results are required, the starch is washed several times with distilled water; these washings are best carried out in the following manner. The starch is mixed with water in a ratio 1:3, stirred vigorously and the particles are allowed to settle. After 30 minutes the supernatant is decanted. Insoluble impurities and the smaller starch particles are removed. After repeated washings the starch is partially dried by sucking on a large Buchner funnel. The starch then is washed two or more times with the buffer in which the electrophoretic run will be carried out. KUNKEL recommends the use of warm buffer solution[14]. After these washings and drying of the starch, it is mixed once more with buffer and stirred until a homogeneous paste is obtained which is poured into the trough. Excess fluid is removed by absorption on thick filter paper. GRANICK AND MAUZERALL[15], who isolated an enzyme which converted *d*-aminolevulinic acid to phosphobilinogen, claim that the recovery could be increased when the starch is pre-washed with 0.25% bovine albumin. In order to prevent evaporation the whole starch block may be packed in water-repellent paper or covered with a sheet of parafilm. When open troughs or boxes are used the surface of the starch may be covered with a layer of molten paraffin wax[16].

The starch block (Fig. 1, a) is connected with the outer compartments of the electrode vessels by means of filter paper strips (Fig. 1, c) folded five or more times and inserted between the outer end of the block and the perspex box. The strips are

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enclosed in parafilm or thin plastic sheets. The connection may also be accomplished by means of plastic sponges, moist cloth, or agar bridges.

Recently RAACKE[60] found that marked changes in the conductivity and pH take place in the starch medium. These changes, caused by the ion-exchange properties of starch can largely be avoided by washing the starch with buffer before electrophoresis.

Insertion of the protein mixture

The box, connected with the electrode vessels, is left for at least 3–4 hours in the cold room for equilibration. Experiments may also be carried out in a refrigerator.

In case the proteins migrate towards the anode, a starch segment 0.5 or 1 cm wide is cut out of the block at 5–7 cm from the cathode side of the box. When the starch is covered with paraffin a window is cut in the wax layer after equilibration in the cold room.

In order to ensure a correct insertion of the mixture under investigation the following procedure is recommended[8]. The protein solution is mixed with starch so

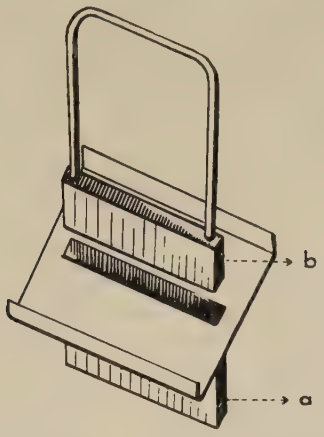

Fig. 2. Stainless steel frame for the insertion of protein-starch mixture (a). Plastic pestle with brass handle (b).

that a paste is obtained of the same consistency as the starch block in the box. Then a steel frame with sharp edges (Fig. 2, a) and the exact proportions of a transverse segment is pressed through the window in the wax layer into the block, the starch within the frame is withdrawn and the inserted segment is pressed by means of a perspex pestle (Fig. 2, b) that fits precisely in the frame. Direct application of the protein by means of a pipette which penetrates the starch block is not advisable on account of irregular flow of the solution which often results in poor separation after the electrophoretic run. Narrow zones may be obtained by careful injection of the sample into the block by means of a hypodermic syringe fitted with a blunt needle[12]. When very small quantities of material have to be separated the solution may be sucked into a piece of Whatman 3MM paper, which has the same cross-section as the starch block. An opening is cut into the starch with a spatula and the wet paper is

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carefully put in by means of two forceps. Direct contact of the paper with the starch is obtained by slight pressure on the starch surface. In this manner tritium-labeled glycyl-alanine was separated from trace amounts of glycine and alanine[17].

Electrophoresis

The circumstances under which electrophoresis in starch medium is carried out differ from case to case. In Table I a survey is given of a number of substances studied, buffers, pH's, voltages, current, and duration of the electrophoretic run.

TABLE 1

Material	*Buffer*	*Ionic strenght (μ) Molarity (M)*	*pH*	*V*	*mA*	*h*	*Ref.*
Normal and pathological serum lipoproteins	Barbital	0.1μ	8.6	100–500	25–60	24	1
	Phosphate	0.05μ	6.5				1
Serum lipoproteins	Barbital	0.1μ	8.6	400	—	24	2
Bovine lens proteins	Barbital	0.025μ	7.8	420	6–8	25	8
Tuberculo-proteins	Barbital	0.1μ	8.6	360	17–20	30	9
Intestinal alkaline phosphatase	Barbital	0.02μ	8.6	200	7–9	12	10
Growth hormone (Somatotropin)	Acetate	0.017*M*	4	175	30	24	11
	Carbonate	0.1*M*	11.2	175	30	72	11
Tobacco mosaic virus	Phosphate	0.01*M*	7.1	380	—	3¼	12
Rabbit reticulocytes	Tris	0.05μ	7.65	270	90	27	15
Chicken erythrocytes	Phosphate	0.13μ	6.9	300	80	19	15
Glycyl-alanine (Tritium-labeled)	Acetate	0.02*M*	3.9	180	4	45	17
β-Galactosidase	Pyrophosphate	0.025*M*	8.5	370	11	16¼	18
Bacterial RNA	Tris	0.015μ	7.6	5.5V/cm	—	15	19
Staphylococcal cell extracts	Tris	0.05*M*	7.6	600	—	4½	26
Chymotrypsinogen	Cacodylate	0.1μ	6.6	3.2V/cm	—	65	27
α-Corticotropin	Acetate	0.1μ	4.0; 5.2		—		28
	Cacodylate	0.1μ	6.6; 7	200	—	24	28
	Barbital	0.1μ	8.3		—		28
Inorganic anions	Acetate	—	7	300	5–15	1	29
Inorganic cations	Lactate	—	3.5; 6.5	800	10–30	½	29
	Phthalate	—	4.5				29
Thyroid stimulating hormone	Phosphate	0.1μ	7.4	300–600	30–50	16–22	31
^{14}C-labeled plasma protein	Barbital	—	—	400–500	24–32	24–36	37
Diphteria antitoxin	Barbital	0.1μ	8.6	500	50–60	18–20	38, 39
Follicle stimulating hormone	Acetate	0.1μ	4	440	10	20	40
Albumin, α-globulin	Barbital	—	8.6	112	27	36	43
Prolactine S. acetamide	Carbonate	0.1*M*	11.2	3.5V/cm	—	48	46
Mammalian tyrosinase	Barbital	0.1μ	8.5	300	11	19	48
Trypsin	Acetate	0.1*M*	4	300	—	10	49
Ribonucleoproteins	Phosphate	0.1μ	9	350–435	—	5¾	50
	Phosphate	0.08μ	8.2	150	—	12	50
Proteins from microsomes	Phosphate	1.5*M*	7	225	11–13	12	53
Proteins from mitochondria	Phosphate	0.05*M*	7.4	6V/cm	20	16–18	55

Influence of temperature

When heat-labile substances are under investigation effective cooling often becomes a serious problem. Control experiments carried out in our laboratory showed a heat effect as indicated in Fig. 3. Working in a cold room without further precautions is not sufficient in itself. The rise of temperature, illustrated in Fig. 3, may be restricted

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to 3° if the temperature of the cold room is kept at —4° during electrophoresis. According to PAIGEN[12] 10 watt over a 24 hours period may be applied if the block is cooled with circulating ice water. The use of a water-jacketed box is then necessary. ROTMAN AND SPIEGELMAN[18] obtained efficient cooling by having the trough in contact both from above and below with lead bricks such as are commonly used for radioactive shielding.

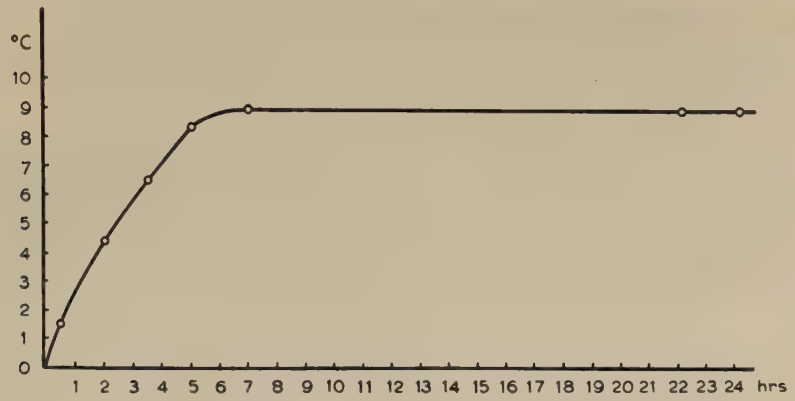

Fig. 3. Rise of temperature in a 40 × 2 × 4 cm starch block during electrophoresis at 200 V, 6–8 mA; in phosphate buffer 0.025 μ. Temperature of the cold room: 2° C.

Location of substance

After the electrophoretic run, the paraffin layer, the parafilm or perspex cover is carefully removed and a strip of Whatman paper is pressed against the starch surface. It is dried for 5 minutes at 80–100°. In case starch particles adhere to the strip they are removed with a soft brush. The strip then is coloured with Amido Black or any other protein dye. In this manner one is able to find the approximate position of protein components which is important mainly for preparative work when sectioning of the whole block is not necessary. The position of nucleic acids is determined by observation of a "print" on Whatman 3MM filter paper under ultraviolet light at 260 mμ[19]; protein, however, will interfere. Radioactive substances may be detected by examination of the paper under a strip counter. Examination of the starch block under ultraviolet light may also be useful when the material shows fluorescence. The most exact location of components is realized by cutting the block, transferring the starch segments to centrifuge tubes, followed by elution and protein estimation in the supernatant after short centrifugation. Settling of the starch without centrifugation is not advisable on account of possible interference of very small particles with protein or RNA determination. The run of sera can be followed by mixing them with a quantity of stain which is bound to the albumin fraction[16].

Cutting

CARLSON[3] already noted that it is difficult to cut the starch with precision. According to our experience errors of 10–20% may arise when segments of 0.5 cm are cut in the way originally described by KUNKEL AND SLATER[2]. The method of PAIGEN[12] too gives

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results of insufficient exactness when proteins, the mobilities of which differ only little, are under investigation.

In order to circumvent these troubles a calibrated box permitting more exact cutting was originally used by us[8]. A guillotine-like frame (Fig. 4, a) is moved over this box. The frame holds a stainless steel knife with the proportions of the cross-section

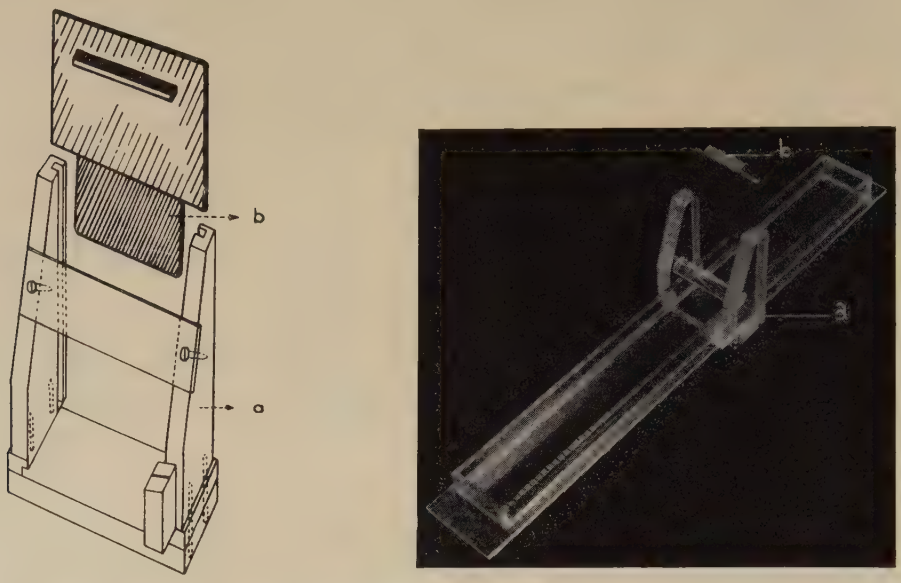

Fig. 4. Cutting device for starch block electrophoresis. (a) Perspex frame. (b) Stainless steel knife.

of the starch block (Fig. 4, b). Although this method improves the slicing technique considerably, small distortions of the starch block may occur which result in poorly reproducible patterns after protein estimation. Furthermore, this procedure is time-consuming, particularly when long boxes are used. Substantial improvement is obtained with the apparatus shown in Figs. 5A and 5B.

A perspex box with removable side walls (Fig. 5, a) fits in a cutting device illustrated in Fig. 5, b. The latter consists of a steel frame joined to a metal plate by means of hinges. Thin stainless steel threads are spanned on the frame at 1 cm intervals[20].

After an electrophoretic experiment is finished the two side walls of the box are removed and the remaining bottom of the box with the starch block is placed in a fixed position on the metal plate of the cutting apparatus. The frame is turned down with the aid of handles (Fig. 5, c). The bottom of the box is supplied with notches at 1 cm intervals into which the threads fit. Cutting of the block is performed in one single manipulation and exactly equal starch segments are obtained.

Elution, protein and nucleic acid determination

The separate starch segments are eluted with ice cold water or buffer solution by stirring and centrifugation during 10 minutes. To obtain optimal recovery the follow-

ing method is recommended. Each starch segment is put on a narrow-stemmed glass filter. Buffer solution added above the packed starch displaces the fluid in the segments by slight suction[14,37]. It has, however, to be considered beforehand whether the protein will not be denatured on the filter surface. The eluates can be concentrated by

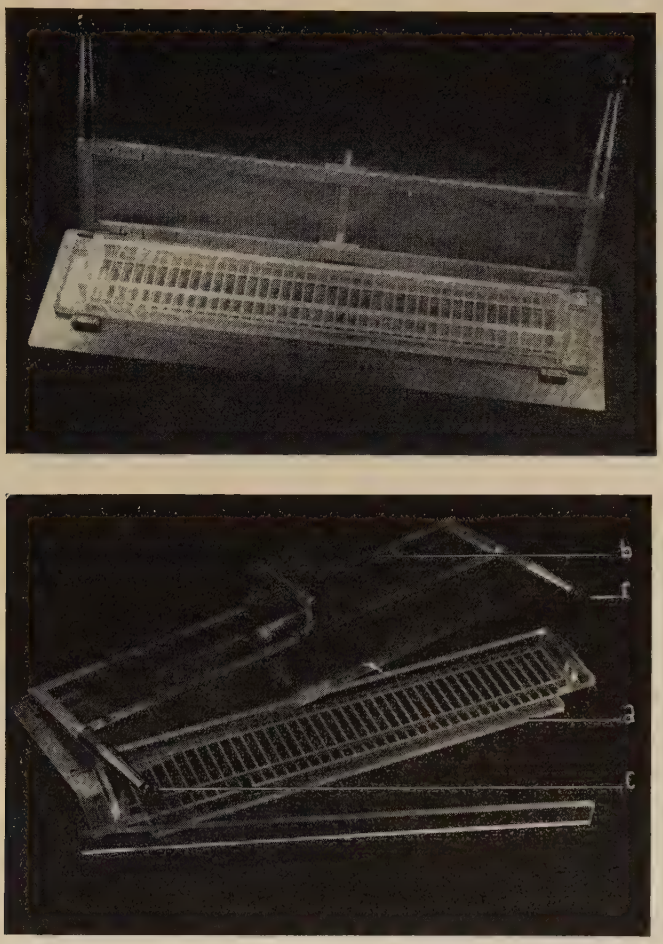

Fig. 5. [A]. Improved cutting apparatus for starch block electrophoresis. [B]. (a) Calibrated box with removable side walls. (b) Cutting device. (c) Handles.

dialyzing against dextran solution or polyvinyl pyrrolidone[21] or by freeze drying. An aliquot of the supernatant fluid is taken from each tube and the protein content is determined according to the method of FOLIN as modified by LOWRY *et al.*[22]. Also direct measurement of the protein extinction at 280 mμ is possible if the solution is completely clear. Direct measurement of nucleic acid contents may be carried out at 260 mμ, but protein or starch traces interfere. Ribonucleic acid and desoxyribonucleic acid are conveniently estimated according to the orcinol[23] and indole[24] methods, respectively. In the case of radioactive material samples of the eluate may

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be counted in a Geiger-Müller or Flow counter[25,26]. (For hormone and enzymatic activity measurements compare the references discussed in the paragraph dealing with applications, p. 54.)

Influence of electrosmosis

In some experiments poor separations are obtained due to a horizontal arrangement of the starch block, or when the buffer solution levels in the electrode vessels are of equal height. Under these circumstances the electrosmotic flow towards the cathode counteracts the electrophoretic migration towards the anode. The starch acquires a negative charge in relation to the buffer, since it is the stationary phase and the buffer moves towards the negative electrode. Dextran, a well known substance

TABLE 2

Medium	$\mu_{el} \times 10^5$	d_{el}/d_{alb}
Filter paper (Whatman 3 MM)	1.5	0.30
Potato starch	2.5	0.62
Washed sea sand	4.3	1.95
Ground glass (35– 60 mesh)	5.1	3.64
Ground glass (150–200 mesh)	5.9	9.87
Soft glass bead (200 mesh)	5.6	6.20
1% Agar	4.7	2.60

for estimating the degree of electrosmosis in paper[13] is not conveniently detected in starch[27]. Table 2 shows the electrosmotic flow for various media expressed in terms of mobility and in relation to the distance of migration of albumin (in barbital buffer 0.1 μ; pH 8.6) as observed by KUNKEL AND SLATER[2]. It appears that electrophoresis on potato starch (with the exception of filter paper) undergoes the smallest influence of the electrosmosis phenomenon.

For the determination of electrophoretic mobilities, μ_{el}, in starch it is necessary to know the displacement of the protein due to electrosmosis, d_{el}[28]. One method to study this is running two known proteins in free as well as in starch electrophoresis; d_{el} then may be calculated from equation (1)

$$\frac{\mu_1}{\mu_2} = \frac{d_1 - d_{el}}{d_2 - d_{el}} \qquad (1)$$

wherein

μ_1 = the mobility of protein 1 in free electrophoresis at the same pH applied in starch electrophoresis,

μ_2 = the mobility of protein 2 in free electrophoresis at the same pH applied in starch electrophoresis,

d_1 = the distance over which protein 1 migrates in the starch block,

d_2 = the distance over which protein 2 migrates in the starch block (under identical conditions of time and field strengths).

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μ_{el} may be computed from the following equations (2) and (3):

$$F = \frac{V}{l} \tag{2}$$

$$\mu_{el} = \frac{d_{el} \cdot l}{Vt} = \frac{d_{el}}{Ft} \tag{3}$$

wherein

l = length of trough (cm), F = field strength, V = average voltage across the ends of the box, t = time (sec).

When the isoelectric point of a protein has to be determined, another calculation may be carried out. The unknown protein is subjected to an electrophoretic run, side by side with a protein of known isoelectric point. In this case equation (4) is used:

$$P_x = P_x\,(\text{obs}) - (P_i\,(\text{obs}) - P_i) \tag{4}$$

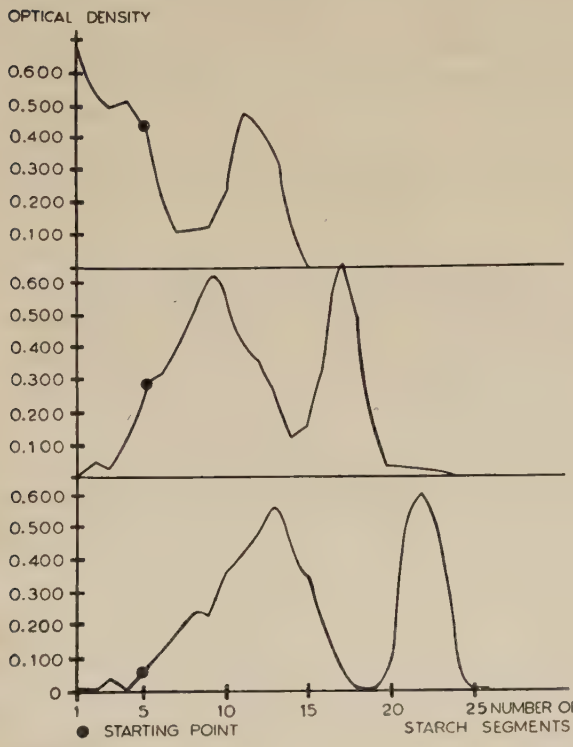
wherein

P_x = the unknown isoelectric point,

P_i = the isoelectric point of the known protein,

P_x(obs) and P_i(obs) = isoelectric points of unknown and known protein, respectively, obtained by plotting the distance of migration against pH.

Fig. 6 demonstrates the effect of electrosmosis on the separation of the water-soluble proteins of the bovine eye lens[8].



(a) Horizontal arrangement of the starch block; buffer vessels at equal height.

(b) Horizontal arrangement of the starch block; difference in levels of buffer = 20 cm.

(c) Vertical arrangement of the starch block; difference in levels of buffer = 20 cm.

Fig. 6. The influence of electrosmosis on the separation of water-soluble lens proteins.

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3. APPLICATIONS

As appears from Table 1 starch block electrophoresis is applied for the separation of various proteins, hormones, enzymes and nucleic acids. Furthermore, attempts have been made to submit inorganic ions to this separation technique[29]. It seems, however, that until now paper or column chromatography give better results for inorganic ions. In addition to the separation and isolation of certain compounds from mixtures the method is repeatedly used as a homogeneity criterion.

(*a*) *Proteins and protein hormones.* The α and β-lipoproteins were readily separated and could be followed by phospholipid and cholesterol analysis on the starch segments. No success was achieved in an attempt at direct staining. Further subdivision of these lipoproteins was obtained with low ionic strength buffers[1,2].

BLOEMENDAL[8] separated α-crystalline from the other water-soluble proteins. This protein migrated as a single boundary when subjected to free electrophoresis at different pH's above and below its isoelectric point[30].

FØNSS-BECH AND LI[11] studied the behavior of somatotropin isolated from the anterior lobe of ox pituitaries. These authors showed that their preparation migrated as a single zone at different pH's. Furthermore, they were able to estimate the isoelectric point.

RAACKE AND LI[28] described the determination of corrected electrophoretic mobilities of different proteins. In their procedure the isoelectric point of corticotropin was found to be 6.6 in monovalent buffers.

POSTEL[31] fractionated serum and pituitary preparations. The uptake of ^{131}I by the chick thyroid gland was used as indicator of TSH activity. The thyroid-stimulating activity of a commercial "pure" TSH preparation appeared to be associated with the protein peak, and was transported primarily with the γ-globulins of human serum when added *in vitro*.

KUNKEL AND WALLENIUS[32] succeeded in showing a new hemoglobin in normal adult blood. This component did not appear clearly in free electrophoresis.

GERALD, COOK AND DIAMOND[33] identified normal oxyhemoglobin and hemoglobin M in a hemolysate obtained from a patient affected with methemoglobinemia.

KUNKEL, TAYLOR AND DU VIGNEAUD[34] were able to carry out a biological assay of an oxytocin fraction isolated after starch block electrophoresis.

MÜLLER-EBERHARD AND KUNKEL[35,36] described a subfractionation of human γ-globulin. The experiments were partially performed in polyvinylchloride as supporting medium. This material is more suitable for carbohydrate analysis, as starch is less satisfactory on account of contamination of carbohydrate derived from the medium.

MILLER AND BALE[37] applied the starch block technique in order to separate ^{14}C-labeled plasma proteins produced by the normal rat and by the isolated perfused liver. The latter incorporated radioactive lysine into the plasma albumin, α-globulin, β-globulin and fibrinogen.

KUHNS[38,39] separated precipitating and non-precipitating skin sensitizing diphteria antitoxin.

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Concentration of FSH activity was achieved by means of starch electrophoresis[40]. Two fractions were obtained representing 42 and 23%, respectively, of the total protein applied. According to the author the low recovery was due to losses of dialyzable material in one of the fractions.

The utility of the described method as purification technique in combination with other isolation procedures was shown by LI and coworkers[41]. They succeeded in obtaining a peptide with high ACTH activity from sheep pituitary. The whole isolation and purification scheme involved: dioxan fractionation, starch electrophoresis, column chromatography and counter current distribution. The same laboratory reported the purification of interstitial cell-stimulating hormone[42]. This hormone was subjected to saline extraction, ethanol precipitation, ammonium sulphate fractionation, column chromatography and zone electrophoresis on starch.

LARSON *et al.*[25] identified thyroxin radiochromatographically in a globulin fraction from serum which was separated by means of zone electrophoresis on starch.

GOLDSWORTHY AND VOLWILER[43] studied the mechanism of protein turnover with labeled plasma proteins of the dog. The starch block technique was applied in combination with the COHN fractionation method[44,45] in order to obtain γ-globulin, fibrinogen, α-globulin and albumin.

Preparations of reduced lactogenic hormone when submitted to zone electrophoresis on starch[46] showed only one peak. The mobility of the reduced hormone appeared to be higher than that of the native prolactin.

SORKIN *et al.*[9] applied a zone transfer technique from starch into glass powder. These investigators are among the few to obtain poor yields with starch block electrophoresis.

(*b*) *Enzymes.* WETTER[47] fractionated proteases from *Mortierella renispora* Dixon-Stewart. Nearly quantitative recovery was obtained for both the activity and nitrogen content. When the separation was performed by means of Whatman No. 3 paper, the yields were extremely poor.

HARRIS AND MEHL[10] achieved approximately 260-fold purification of a crude preparation of alkaline phosphatase from bovine intestinal mucosa. However, the amounts of purified product were only small. The results of these authors indicate that the enzyme may have been present initially in a complexed form and that this complex was disrupted during sequential electrophoretic runs in several starch blocks.

Efforts to remove protein impurities present in chromatographically prepared mammalian tyrosinase did not result in further purification. Approximately 99% of the activity was recovered[48].

LIENER AND VISWANATA[49] separated autolysed trypsine into an active fast moving component and a less active fraction which corresponded to dialyzable split products.

ROTMAN AND SPIEGELMAN[18] investigated extracts of *Escherichia coli* for β-galactosidase activity. One single run could effect a 30-fold purification whereas 80% of the available enzyme could be recovered.

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(*c*) *Ribonucleoproteins and nucleic acids.* ELSON[50] subjected ribonucleoproteins to zone electrophoresis on starch before and after treatment with urea. According to his findings there are indications that hydrogen bonds play an important role in the linkage of RNA with the protein.

PAIGEN[12] applied the method to tobacco mosaic virus strains. Separation into three components was possible.

PARDEE, PAIGEN AND PRESTIDGE[19] studied the electrophoretic behavior of RNA, DNA and protein from *Escherichia coli* and *Bacillus megatheria*. They were able to show that bacterial RNA migrates only in two distincts zone. The electrophoretic patterns obtained depended on the buffers employed, a phenomenon which is also well known in paper electrophoresis[51,52]. Approximately 90% of the nucleic acid and 70% of the protein were recovered.

(*d*) *Proteins from cell fractions.* Lately, starch block electrophoresis is being used also for the study of subcellular fractions. GRANICK AND MAUZERALL[15] described the fractionation of a soluble enzyme fraction from red cells. They observed one main component migrating towards the cathode, which appeared to be hemoglobin, whereas three colorless enzyme fractions moved towards the positive electrode. A total of 30 ml fluid could be fractionated.

ARCOS AND ARCOS[53] made an attempt at detecting structural alterations in liver microsomes during chemical carcinogenesis. Swelling had been used to detect these alterations[54]. The swelling of microsomes from liver tissue was paralelled by the release of a group of soluble proteins not sedimenting at 105,000 *g*. These proteins were resolved into two fractions on starch. Microsomes from hepatoma on the contrary showed low swelling with relatively high protein release. These investigators did not include the proteins of hepatoma in their electrophoretic studies.

WIRTZ AND ARCOS[61] made a comparative study of the electrophoretic pattern of rat liver and hepatoma supernatant fluids. The so-called protein "h" was isolated and its molecular weight, amino acid composition, and bound azo-dye were determined.

BERESOVSKAYA[55] isolated an enzyme from mitochondria which catalyzed the amino acid synthesis from pyruvic acid and ammonia. The active protein was present in a fast migrating fraction which had a higher mobility than had serum albumin. The electrophoretic separation resulted in a 300–350 fold purification of the enzyme as compared with the original liver homogenate.

GALE and coworkers[26] isolated a fast moving fraction from extracts of disrupted staphylococcal cells which were previously incubated with ^{14}C-L-glutamic acid as the only labeled amino acid precursor. The authors provide evidence that this fraction represents an intermediate stage in protein synthesis. If this is so the L-glutamic acid containing intermediate should be very stable as the heat development in a starch block under the conditions of their experiment (20 V per cm, pH 7.6) may be considerable.

BLOEMENDAL AND BOSCH[62] fractionated the ^{14}C-leucine labeled pH5 enzyme from rat liver cytoplasma. An important working condition was air-cooling of the starch block to —5°.

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(*e*) *Homogeneity studies.* RAACKE[27,28,56] used starch electrophoresis for homogeneity studies of ribonuclease, lysozyme, bovine serum albumin, chymotrypsinogen and chymotrypsin. All preparations investigated yielded more than one peak after variation of buffers and the application of a pH-range from 4.7 to 9.1. These results emphasized once more the importance of pH variation. On the other hand one has to realize whether the peaks observed are a proof of heterogeneity or that the substance under investigation was no longer stable at any "extreme" pH in the alkaline or acid range. Such stability boundaries were observed *e.g.* in the case of the lens protein α-crystalline below pH 4 and above pH 8.9[57]. According to RAACKE starch electrophoretical homogeneity experiments may be considered to be as sensitive as the reversible boundary spreading test[58] and superior to the moving boundary method.

WADA *et al.*[59] concluded to the homogeneity of soy bean hemagglutinin after finding one peak at one pH. In this connection it must be mentioned that a number of workers are as yet satisfied with one single pH value. The conclusion reached then can only be that the protein is *electrophoretically* homogeneous at *this* pH.

4. CONCLUSION

Starch block electrophoresis is of growing importance as a useful tool in protein chemistry for analytical studies as well as for isolation on preparative scale. However, reproducible results will be obtained only if the working methods are exactly standardized. In a few cases polyvinylchloride as a supporting medium may be more satisfactory than starch; working conditions, however, remain identical.

Poor separation may sometimes be the result of horizontal arrangement of the troughs. Gravity then may cause accumulation of protein in the lower starch layers, leading to uneven mobilities at different levels of the block. Furthermore, the electrosmotic flow may counteract the electrophoretic migration. Vertical position of the starch block is therefore preferable.

In summary, the starch block technique opens up the possibility for separation of various protein mixtures; it is often successfully applied in combination with other isolation methods and, in some cases, gives good results where other fractionation procedures fail.

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PAPER CHROMATOGRAPHY OF DINITROPHENYLAMINO ACIDS

GÉRARD BISERTE, JAMES W. HOLLEMAN*,
JANINE HOLLEMAN-DEHOVE AND PIERRE SAUTIÈRE
Laboratory of Biological Chemistry, Faculty of Medicine and Pharmacy, Lille (France)

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1. INTRODUCTION

The dinitrophenylamino acids (DNP-amino acids) are derivatives frequently employed in the chemistry of proteins. At laboratory temperature and in a slightly alkaline medium, 1-fluoro-2,4-dinitrobenzene (FDNB) readily reacts with the free α-amino groups of an amino acid, a peptide or a protein, to give the dinitrophenylamino derivatives. In the course of this coupling reaction, the dinitrophenyl radical can also become attached to the thiol group of cysteine, the phenol group of tyrosine, the

* Present address: Department of Anatomy, College of Medicine, Wayne State University, 1401 Rivard St., Detroit 7, Mich. (U.S.A.).

ω-amino groups of the diamino acids, as well as to the imidazole nucleus of histidine. Arginine and the amides of dicarboxylic acids give a monodinitrophenyl derivative of their α-amino group.

Very many of the dinitrophenylamino acids (DNP-amino acids) are ether-soluble. Some of them, however, such as α-mono-DNP-arginine, DNP-cysteic acid, α-DNP-histidine, ε-mono-DNP-lysine, O-DNP-tyrosine, S-DNP-cysteine, and imidazole-DNP-histidine, are water-soluble.

Determination of the amino acid, containing a free α-amino group, in a terminal position in a peptide chain is very often effected by SANGER's dinitrophenylamino acid method.

The amino acids of a total hydrolysate or of an enzymic hydrolysate (produced by the action of carboxypeptidase or leucine aminopeptidase) of a protein can also be identified and determined as their dinitrophenyl derivatives.

Thus the identification and determination of DNP-amino acids constitute fundamental problems of analysis, chromatography being the principal separation method.

Numerous techniques of chromatography on columns have been described: chromatography on silica gel (SANGER[76], PORTER AND SANGER[67]); on buffered silica gel (BLACKBURN[12], MIDDLEBROOK[57]); on Kieselguhr (MILLS[58], BRAUNITZER AND REUTHER[16]); on buffered Hyflo-Super-Cel (BELL *et al.*[7]); on silicic acid, Celite (GREEN AND KAY[3]). But it is much more convenient to employ partition chromatography on paper. Numerous solvent systems have been proposed, which are more or less efficient. At present, the separations are readily achieved with the aid of various types of two-dimensional chromatography, which we shall describe later in this article. Methods of reversed phase chromatography have also been published: on chlorinated rubber (PARTRIDGE AND SWAIN[63]), on acetylated paper (BURTON[18]).

2. PREPARATION OF THE DINITROPHENYLAMINO ACIDS

The preparation of the DNP-amino acids used as reference substances has been described in the publications of SANGER[76], PORTER AND SANGER[67], LEVY AND CHUNG[45], FRAENKEL-CONRAT, HARRIS AND LEVY[28], and RAO AND SOBER[69].

The preparation and physical properties of numerous ether-soluble DNP-amino acids are recorded in the articles by RAO AND SOBER[69] and FRAENKEL-CONRAT, HARRIS AND LEVY[28].

(*a*) *General methods of synthesis*

Two convenient methods of synthesis may be advocated:

1. *Method of* LEVY AND CHUNG[45]

Coupling is carried out in an aqueous medium at 40°. The amino acid (10 mmoles) and anhydrous sodium carbonate (2 g) are dissolved in 40 ml of water at 40°. The fluorodinitrobenzene (10 mmoles) is added and the mixture vigorously agitated, the temperature being maintained at 40°. The small drops of fluorodinitrobenzene in

suspension disappear after about half an hour, which marks the end of the reaction. Acidification (3 ml concentrated hydrochloric acid) of the orange solution precipitates the DNP-amino acid, crystallization of which is induced by scratching. For the recrystallization of the DNP-amino acid special solvent mixtures are used (see Table 1).

TABLE 1

SOLVENTS FOR THE PURIFICATION AND CRYSTALLIZATION* OF THE DNP-AMINO ACIDS
(According to RAO AND SOBER[69])

Ether–petroleum ether (b.p. 30–70°)	L-Alanine, DL- and L-α-aminobutyric acid, DL-and L-valine, DL- and L-glutamic acid, L-norvaline, L-isovaline, DL- and L-leucine, DL-, D- and L-*allo*isoleucine, DL- and L-threonine, DL- and L-*allo*threonine, DL-methionine, DL-ethionine, L-cystine, S-benzyl-L-cysteine, L-phenylalanine, DL- and L-proline, hydroxy-L-proline, DL-pipecolic acid.
Aqueous methanol	Glycine, DL- and L-serine, L-asparagine, L-glutamine.
Aqueous acetone	γ-Hydroxy-L-α-aminobutyric acid, β-hydroxy-L-α-aminocaproic acid, L-α,γ-diaminobutyric acid, L-arginine, di-DNP-histidine.
Acetone–ether	O,N-Di-DNP-L-tyrosine, L-tryptophan, di-DNP-L-lysine
Acetone–petroleum ether	Di-DNP-L-ornithine
Aqueous ethanol	β-Alanine

* In general, the DNP-DL-amino acids crystallize more easily than the corresponding derivatives of the L series, particularly glutamic acid, methionine, leucine and tyrosine.

The bis-dinitrophenyl derivatives (cystine, tyrosine, lysine, histidine) require two millimoles of fluorodinitrobenzene for one of the amino acid. The quantity of sodium carbonate is increased to 4 g in the case of cystine and lysine and to 3 g in the case of tyrosine, histidine, aspartic acid and glutamic acid.

2. *Method of* RAO AND SOBER[69]

Coupling is effected by shaking the amino acid with fluorodinitrobenzene in the presence of a slight excess of sodium bicarbonate for 2 to 5 h in 50 % ethanol at room temperature (it is recommended that this reaction as well as all stages of the preparation be carried out in the dark). The alcohol is eliminated at room temperature and the excess of fluorodinitrobenzene extracted by shaking three times with ether. The aqueous solution is acidified with 6 *N* hydrochloric acid until the reaction is distinctly acid. The precipitate or oil that separates out is washed several times with small quantities of ice water.

(*i*) *DNP-amino acids that precipitate as an oil.* The following method is applicable to the following amino acids of the L-series: valine, alanine, α-aminobutyric acid, norvaline, isovaline, threonine, *allo*-threonine, leucine, isoleucine, *allo*isoleucine, hydroxyproline, phenylalanine, aspartic acid, cystine, as well as DL-glutamic acid, DL-proline, DL-threonine, DL-methionine, DL-ethionine, DL-α-aminobutyric acid, DL-valine, DL-pipecolic acid, D-threonine.

The substance is dissolved in a large volume of actone and the solution dried

over anhydrous sodium sulphate. After filtration, the solution is concentrated to a small volume. An equal volume of benzene is added to the acetone solution and the DNP-amino acid precipitated by an excess of petroleum ether (b.p. 30–75°). The derivative is dried in a current of air, dissolved in ether and precipitated with petroleum ether. The ether–petroleum ether process is repeated several times, until the DNP-amino acid crystallizes at a low temperature.

(*ii*) *DNP-amino acids that are precipitated as solids.* Glutamine, L-serine, L-tyrosine, L-tryptophan, L-arginine, L-histidine, L-α,γ-diaminobutyric acid, L-ornithine, L-lysine, γ-aminobutyric acid and L-asparagine.

The precipitates are washed with ice water and crystallized by using appropriate solvents (see Table I).

(*b*) *Methods of synthesis in special cases*

1. *DNP-L-glutamic acid*

(*i*) *Procedure of* RAO AND SOBER[69]. Owing to the difficulty of crystallization, the authors start with DNP-glutamine, which crystallizes readily. This is hydrolysed for one night with 10 times the volume of 6 *N* hydrochloric acid and heated on a water bath until the product dissolves. The solution, cooled to room temperature, is then placed in a refrigerator until a yellow viscous oil separates out, which crystallizes after being kept in the cold for several weeks. The DNP-amino acid, washed with water and vacuum dried over P_2O_5, appears in the form of a yellow hygroscopic solid.

2. *Derivatives of histidine**

(*i*) *α-Mono-DNP-histidine* (a compound showing a positive Pauly reaction and giving no ninhydrin reaction). The derivative monosubstituted at the α-position of histidine is obtained by allowing 0.5 molecule (or less) of fluorodinitrobenzene to react with 1.0 molecule of histidine. The method adopted by RAMACHANDRAN AND MCCONNELL[68] is as follows: 1.917 g (0.01 mole) of L-histidine monohydrochloride and 8.4 g of sodium bicarbonate are dissolved in 200 ml of water, and 0.453 g (0.0025 mole) of fluorodinitrobenzene in 25 ml of ethanol is added. After leaving for 1 h at room temperature the volume is reduced under vacuum to 50 ml and the pH adjusted to 6.5 by carefully adding conc. hydrochloric acid. The precipitate is filtered off and recrystallized by dissolving in the minimum volume of aqueous ethanol. The shiny yellow crystals obtained after 18 h at a low temperature are collected and dried under vacuum at 60°. M.p. (not corrected): 278–280° (decomposition). See also ZAHN AND PFANNMÜLLER[97].

(*ii*) *Di-DNP-histidine* (see above). The dinitrophenyl derivative is obtained by using an excess of fluorodinitrobenzene (2.5 moles per mole of histidine according to RAMACHANDRAN AND MCCONNELL[68]). Crystallization is effected with aqueous acetone.

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* See also ZAHN AND PFANNMÜLLER[99].

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(*iii*) *Imidazole-DNP-histidine* (colourless compound giving a brown colour with ninhydrin and a negative Pauly reaction). Synthesis is effected by starting from α-acetylhistidine (method of BERGMANN AND ZERVAS[8]). After dinitrophenylation, α-acetyl-mono-DNP-imidazole-histidine is hydrolysed with boiling 20% hydrochloric acid (MARGOLIASH[54]). Imidazole-DNP-histidine can also be obtained by hydrolysing the peptide DNP-histidylhistidine. But the hydrolysate also contains di-DNP-histidine and sometimes α-mono-DNP-histidine.

3. *O-DNP-tyrosine* (SANGER's method)[77]

N-Acetyl-L-tyrosine (0.55 g) (DU VIGNEAUD AND MEYER[26]) is treated in a bicarbonate solution for 4 h with 2.0 g of chlorodinitrobenzene dissolved in ethanol. After acidification, an oil separates out and partially crystallizes. The N-acetyl-O-DNP-tyrosine is hydrolysed by refluxing with 20% hydrochloric acid for 3 h. After cooling and evaporating to a small volume, a precipitate is obtained which is filtered off, dissolved in hot dilute nitric acid and neutralized with pyridine while still hot. The O-DNP-tyrosine crystallizes in white needles containing 1 molecule of water of crystallization (m.p. 202°). See also FRITZE AND ZAHN[29b].

4. *α-Mono-DNP-arginine* (PORTER AND SANGER[67])

Coupling is carried out in aqueous ethanol. 3 moles of amino acid and 1.1 g of sodium bicarbonate are dissolved in 14 ml of water and then 1.1 g (6 mmoles) of fluorodinitrobenzene in 28 ml of ethanol are added. The mixture is agitated for 2 h at room temperature and concentrated to eliminate the ethanol.

After removal of the ethanol, the residue is treated with water in which DNP-arginine is insoluble. After filtration it is washed with ethanol and ether, and recrystallized by means of a dilute solution of hydrochloric acid neutralized with ammonia.

5. *Derivatives of ornithine*

(*i*) *α-Mono-DNP-ornithine.* α-DNP-δ-benzoyl-ornithine, obtained by dinitrophenylation of δ-benzoyl-ornithine (SANGER[77]), is hydrolysed.

The monohydrochloride of DL-ornithine (0.3 g) (prepared from the dihydrochloride by RIVARD's method[73]) is converted into its copper complex (see Section 2, b, 5, ii, p. 64) and the cooled solution is benzoylated in the usual manner with 0.32 ml of benzoyl chloride in the presence of 6 ml of *N* NaOH. The insoluble benzoyl derivative is separated by filtration, suspended in 2 ml of water and treated with hydrogen sulphide. The solution is brought to boiling and filtered while it is hot. After concentration of the filtrate to about 5 ml, δ-benzoyl-DL-ornithine crystallizes out (0.14 g). It is suspended in 2 ml of water containing 0.3 g of sodium bicarbonate and agitated for 2 h with a solution of 0.2 ml of fluorodinitrobenzene in 4 ml of ethanol. After elimination of the ethanol under reduced pressure, the excess of fluorodinitrobenzene is removed with ether and the solution acidified. α-DNP-δ-benzoyl-DL-ornithine is precipitated immediately in the form an amorphous solid (yield, 0.2 g). The benzoyl

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group is removed by heating with a mixture of 2 ml of 10 *N* HCl and 2 ml of pure glacial acetic acid, for 4 days at 105° in a sealed tube under vacuum. After cooling, the solution is evaporated to dryness and the residue dissolved in water. The non-hydrolysed product (sometimes in considerable quantity) is extracted with ethyl acetate and, after neutralization of the aqueous solution with pyridine, α-DNP-ornithine crystallizes out (m.p. 227°).

Instead of employing the above technique, we prefer to dinitrophenylate δ-carbobenzoxy-DL-ornithine (see below, section 6 (*i*), preparation of ε-carbobenzoxy-lysine) and remove the carbobenzoxy group by hydrolysis with 5.6 *N* hydrochloric acid at 100° for 24 h.

(*ii*) *δ-DNP-L-ornithine.* This is prepared by dinitrophenylation of the copper complex of the monohydrochloride of L-ornithine (SANGER[77]).

0.1 g of L-ornithine monohydrochloride is dissolved in 5 ml of hot water and treated with an excess of copper carbonate. After filtration, the solution is evaporated to a volume of about 2 ml and 0.3 g of sodium bicarbonate and a solution of 0.2 ml of fluorodinitrobenzene in 4 ml of ethanol are added. The mixture is agitated for 2 h at room temperature. The copper complex of δ-DNP-ornithine is precipitated in the form of a greenish powder. After filtration and dissolution in dilute hydrochloric acid, the solution is treated with hydrogen sulphide and filtered through charcoal. After concentration, the hydrochloride of δ-DNP-L-ornithine crystallizes. It is recrystallized after dissolution in *N* hydrochloric acid (m.p. 228°, with decomposition).

(*iii*) *Di-DNP-ornithine.* This is easily obtained by the classical methods (excess of fluorodinitrobenzene).

6. *Derivatives of lysine*

(*i*) *α-Mono-DNP-L-lysine.* This can be obtained by dinitrophenylation of ε-benzoyl-lysine, of ε-acetyl-lysine or of ε-carbobenzoxy-lysine, followed by a specific hydrolysis of the group protecting the ε-amino radical.

ε-Benzoyl-lysine (SANGER[76]). The method is not very convenient.

ε-Acetyl-lysine. This derivative is prepared by the method of NEUBERGER AND SANGER[6]. After dinitrophenylation of ε-acetyl-lysine, α-DNP-ε-acetyl-lysine is extracted with ether from the acid solution. The acetyl group is removed by treating with 6 *N* hydrochloric acid for 2 h under 15 lb. steam pressure. The acid is expelled under reduced pressure and α-DNP-lysine precipitated with pyridine (FOLK[27]) (m.p. 270°, with decomposition).

ε-Carbobenzoxy-L-lysine. This derivative is prepared either by the method of BERGMANN *et al.*[9] or by that of NEUBERGER AND SANGER[61].

The monohydrochloride of lysine (1.8 g) (prepared from the dihydrochloride by the method described by RICE[72]) is treated in hot aqueous solution with an excess of copper carbonate. The excess of copper carbonate is removed by filtration. 5 ml of 2 *N* NaOH are added and the dark blue solution is cooled in ice. Benzoyl chloride (2 ml) and 2 *N* NaOH (10 ml) are added in 10 portions during 30 min with agitation and cooling, care being taken that the solution does not become too alkaline. The

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copper complex of ε-carbobenzoxy-lysine is precipitated. After filtration, it is washed with ethanol and with water. Then it is suspended in 20 ml of water and hydrogen sulphide is passed through. The solution, brought to boiling, is filtered while hot and ε-carbobenzoxy-lysine crystallizes in fine needles. It is recrystallized from an aqueous solution.

The dinitrophenylation of ε-carbobenzoxy-lysine is effected by the usual methods, and α-DNP-ε-carbobenzoxy-lysine is hydrolysed by means of 5.6 *N* hydrochloric acid, for 16 h at 100°.

(*ii*) *ε-Mono-DNP-lysine.* This is prepared by dinitrophenylation of the copper complex of L-lysine monohydrochloride (PORTER AND SANGER[67]).

L-Lysine (0.5 g) is dissolved in 10 ml of water and copper carbonate is added slowly to the boiling solution. The excess of copper carbonate is removed by filtration. An excess of sodium bicarbonate and a solution of 1.5 g of fluorodinitrobenzene in 20 ml of ethanol are added. The mixture is agitated for 2 h at room temperature. The greenish yellow precipitate is collected by filtration and washed with water, ethanol and ether. It is suspended in 5 ml of water and a sufficient quantity of *N* hydrochloric acid is added to obtain a clear solution. The solution is cooled in ice and a current of hydrogen sulphide is passed through for 2 min. A small amount of charcoal is added and the mixture is immediately filtered. The filtrate is rapidly evaporated to dryness under reduced pressure.The product is crystallized from a solution in 20 % hydrochloric acid.

(*iii*) *Di-DNP-lysine.* This is easily obtained by the classical methods (see process of LEVY AND CHUNG, section 2, a, 1, p. 60). It is recrystallized from aqueous methanol.

7. Derivatives of cystine

(*i*) *Mono-DNP-cystine* (BETTELHEIM[10]). 1.2 g of L-cystine and 2 g of sodium carbonate are dissolved in 50 ml of water and then 0.9 g of fluorodinitrobenzene dissolved in 5 ml of ethanol is added. After agitation for 20 min the pH is adjusted to 7 with hydrochloric acid and the unreacted cystine precipitated. The solution is again acidified and concentrated. Sodium chloride is precipitated with acetone. After desiccation, the residue is washed with ether and crystallized from an aqueous solution (m.p. 187°, not corrected).

(*ii*) *Di-DNP-cystine.* This is prepared by the classical methods. Crystallization can be effected by means of aqueous ethylene glycol mono ethyl ether. It is recrystallized from a solution in dilute acetic acid (PORTER AND SANGER[67]).

(*iii*) *S-DNP-cysteine.* After dinitrophenylation of reduced glutathione, the total hydrolysate contains S-DNP-cysteine (water-soluble) and DNP-glutamic acid (ether-soluble) (HAUSMANN, WEISIGER AND CRAIG[32]).

(*iv*) *DNP-cysteic acid* (potassium salt). To obtain this water-soluble dinitrophenyl derivative it is convenient to oxidize di-DNP-cystine with performic acid (BETTELHEIM[10]).

0.2 g of di-DNP-L-cystine is oxidized with 10 ml of performic acid (9 ml of pure formic acid + 1 ml of hydrogen peroxide to 110 volumes) for 30 min. The solution is

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evaporated to dryness and the residue is dissolved in a little water. The solution is adjusted to pH 6 with KOH, and on the addition of ethanol and ether crystallization occurs.

8. For *N,N'-di-DNP-mesolanthionine* see ZAHN AND PFANNMÜLLER[98], and for coupling reactions with 1,5-difluoro-2,4-dinitrobenzene see ZAHN AND MEIENHOFER[96].

3. DINITROPHENYLATION OF A PROTEIN HYDROLYSATE (Table 2)

(*a*) *Hydrolysis of the protein*

3–5 mg of protein are hydrolysed in a sealed tube under vacuum with 1–2 ml of 5.7 *N** hydrochloric acid at 105° for 24 h**. The hydrochloric acid is very carefully removed.

(*b*) *Coupling with fluorodinitrobenzene; extraction of the dinitrophenyl derivatives*

Various methods of coupling the amino acids with fluorodinitrobenzene have been described. The reaction can be carried out in either an aqueous or an aqueous alcoholic medium.

1. *Coupling in aqueous medium* (LEVY *et al.*[46])

(*i*) *Reaction procedure.* The hydrolysate (3–5 mg in 3 ml) is placed in a synthesis cell (see Fig. 1). 0.1 ml of 3.1 *N* potassium chloride is added and the contents are adjusted to pH 9.0 (with about 40 μmoles of 0.2 *N* NaOH). The solution is saturated with fluorodinitrobenzene at 40° by vigorous agitation with a slight excess (about 0.1 ml) of the reagent. The pH is maintained at 9 for 80 min by intermittent additions of 0.2 *N* NaOH. This operation can be conveniently carried out by means of an autotitrator (JACOBSEN AND LEONIS[37])

The kinetics of the reaction can be followed by measuring the consumption of sodium hydroxide (Fig. 2). Given that the fluorodinitrobenzene is in excess, the reaction medium remains saturated with the reagent and the formation of dinitrophenol*** is constant during the reaction (0.044 μmole/ml/min) (LEVY[43]). Extrapolation to zero time of the final slope of the curve, resulting from the formation of dinitrophenol, indicates the number of μmoles of sodium hydroxide consumed during the coupling reaction with the amino acids (Fig. 2).

FRAENKEL-CONRAT AND SINGER[29] have also described a coupling reaction in

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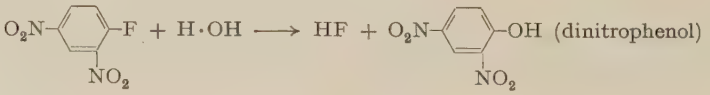
* The hydrochloric acid is prepared by distilling an azeotropic mixture of hydrochloric acid and water 3 or 4 times in a glass apparatus.

** The hydrolysis usually requires 24 h; it might be useful to determine the amino acids in several hydrolysates obtained after different periods of hydrolysis (*e.g.* 24 and 48 h).

*** Besides the coupling reaction with the amino acids (FDNB + H_2N-CH(R)-COOH → DNP-HN-CH(R)-COOH + HF), alkaline hydrolysis of the reagent also occurs.



5 % carbonate buffer at pH 9.3 during 3 hours at 40°. The quantity of fluorodinitrobenzene is 15 μl per 2 mg of amino acids.

(*ii*) *Extraction of the dinitrophenyl derivatives.* When the coupling reaction is terminated, the contents of the cell are emptied quantitatively into a separating funnel and extracted with peroxide-free ether* (2 to 4 times with 5 ml) to remove the excess of fluorodinitrobenzene.The mixture is then acidified (0.5 ml of 5 *N* HCl)

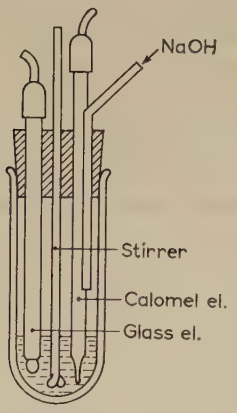


Fig. 1. Synthesis cell for the dinitrophenylation of amino acids. Glass el. = glass electrode; Calomel el. = calomel electrode.

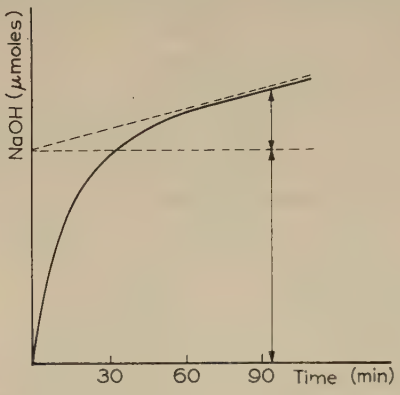


Fig. 2. Graph showing the consumption of sodium hydroxide during dinitrophenylation. Extrapolation to zero time of the final slope of the curve, which results from the formation of dinitrophenol, indicates the number of μmoles of sodium hydroxide consumed during the coupling reaction with the amino acids.

and the ether-soluble DNP-amino acids are extracted with ether (5 times with 5 ml). LEVY[43] takes aliquot parts of the ether solution (3 portions of 2 ml) and evaporates them to dryness. The dry residues are dissolved in acetone and their solution is placed on sheets of Whatman No. 1 paper. We prefer the following procedure:

The ethereal phase is concentrated carefully to a small volume, in glass dishes; the ethereal layer, which floats above some droplets of water, is poured quantitatively into special receptacles (Fig. 3) for the sublimation of the dinitrophenol (our own technique, derived from that of MILLS, see below).

The aqueous phase remaining after extraction of the ether-soluble DNP-amino acids still contains α-mono-DNP-arginine and α-DNP-histidine (and perhaps DNP-cysteic acid)**. It may be treated in various ways:

According to LEVY[43], the aqueous phase is diluted to 10 ml and aliquot parts (3 times 1 ml) are evaporated to dryness. After dissolution in a known volume of acetone, the solution of DNP-amino acids is placed on sheets of Whatman No. 1 paper.

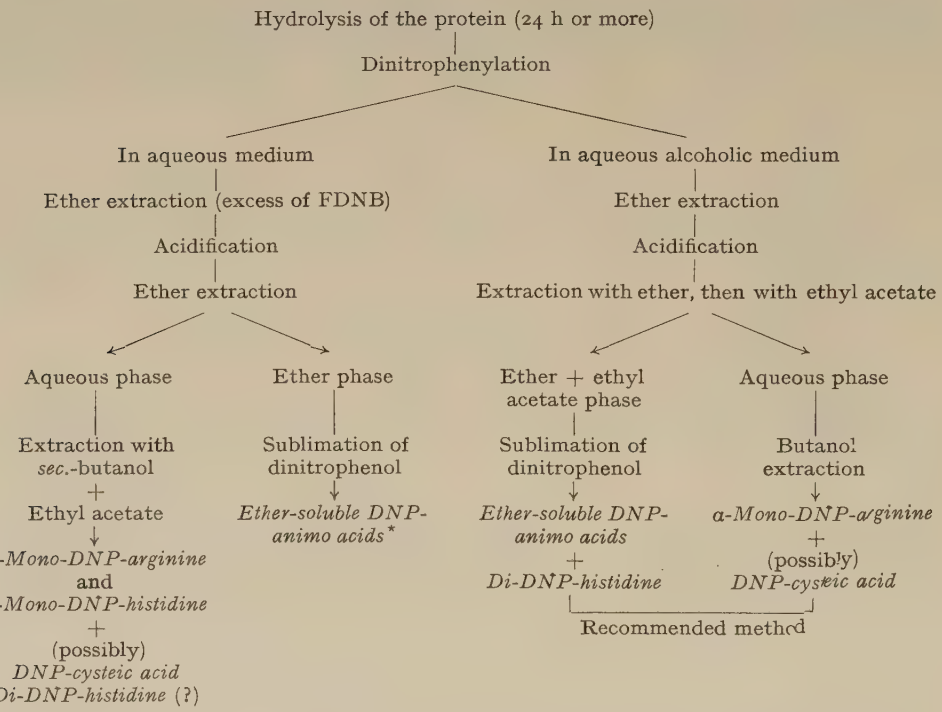
We prefer, like KOCH AND WEIDEL[39], to extract the remaining aqueous phase

* To avoid the conversion of DNP-methionine into DNP-methionine sulphone, it is essential to use ether that is free from peroxides. To remove peroxides the ether is distilled over stannous chloride, then washed with a 20 % solution of sodium carbonate and with water; it is kept over powdered ferrous sulphate, away from light. The ether can also be freed from peroxides by passing it through a column of activated alumina (DASLER AND BAUER[21]).

** DNP-cysteic acid occurs in the total hydrolysates of a protein that has been oxidized with performic acid or in the total hydrolysates that have been oxidized with performic acid.

TABLE 2

DETERMINATION OF AMINO ACIDS AS THEIR DINITROPHENYL DERIVATIVES
(LEVY'S method[43])



* *N.B.*: It should be noted that very small amounts of di-DNP-histidine may accompany the ether-soluble DNP-amino acids.

several times with a mixture of equal volumes of *sec.*-butanol and ethyl acetate. After desiccation, the water-soluble DNP-amino acids are dissolved in a known volume (3 ml, for example) of acetone*. Aliquot parts of this solution (50 to 150 μl) are placed on Whatman No. 1 paper.

2. Coupling in an aqueous alcoholic medium

To achieve total conversion of histidine into di-DNP-histidine, while avoiding the formation of α-mono-DNP-histidine (see above), FRAENKEL-CONRAT AND SINGER[29] carry out the reaction with fluorodinitrobenzene in an aqueous alcoholic medium according to the following technique:

Coupling is carried out in a medium containing 0.7 % of sodium bicarbonate, 1.7 % of fluorodinitrobenzene and 67 % of ethanol for 80 min at 20–25°. The excess of fluorodinitrobenzene is extracted with ether after evaporation of most of the alcohol.

* To facilitate the dissolution of the water-soluble DNP-amino acids 0.9 ml + acetone *N* 0.1 ml *N* hydrochloric acid may be used.

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The reaction medium is then acidified (to about pH 1–2) and extracted, first with peroxide-free ether and then with ethyl acetate, which makes possible the quantitative extraction of di-DNP-histidine. The remaining aqueous phase contains only α-mono-DNP-arginine and perhaps DNP-cysteic acid.

We prefer the following method:

The hydrolysate (10 mg of protein) is dissolved in 5 ml of double distilled water, which is brought to and maintained at 40°. The pH is adjusted to 9 with *N*/15 NaOH and 0.2 ml of fluorodinitrobenzene is added. The solution is stirred for 15 min at 40° while the pH is maintained at 9. Ten ml of absolute ethanol are then added and stirring is continued for 90 min at 40°, the pH still being kept at 9. After the reaction, the alcohol is removed by evaporation in a current of cold air. The excess of fluorodinitrobenzene is extracted several times (5 to 10 times) with peroxide-free ether. The medium is acidified (1 ml of concentrated hydrochloric acid) and again extracted with peroxide-free ether (5 extractions) and then with ethyl acetate (3 extractions). The residual aqueous phase is extracted with a mixture of equal parts of ethyl acetate and *sec.*-butanol (3 extractions).

The ether and ethyl acetate extracts are collected and evaporated to dryness, and the dinitrophenol present in this phase is eliminated by sublimation (see below for a description of the technique employed). The DNP-amino acids are then dissolved in 2 ml of acetone.

The butanol extracts are also evaporated to dryness, then dissolved in acetone (1–2 ml). Aliquots (corresponding to 0.1–0.3 mg of protein) of the acetone solutions of the residues of the ether-soluble and water-soluble DNP-amino acids are placed on sheets of Whatman No. 1 paper.

(c) *Sublimation of dinitrophenol*

The classical method of eliminating dinitrophenol is by sublimation (MILLS[58]). The sublimation apparatus used by the authors is shown in Fig. 3. This apparatus is

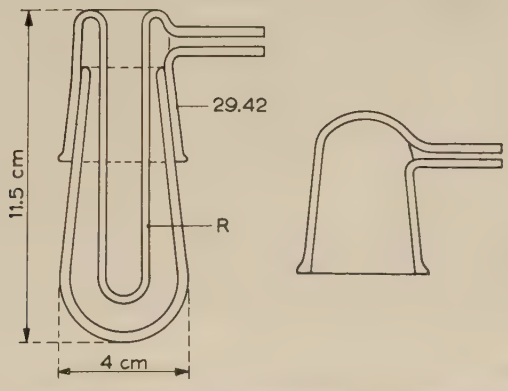

Fig. 3. Apparatus employed for the sublimation of dinitrophenol. A standard 29-42 ground glass joint is used. The cooling finger (R) is filled with the acetone + carbon dioxide snow mixture. The glass cap at the right-hand side of the figure facilitates the elimination of droplets of water after evaporation of the ether solution and before the sublimation is carried out.

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designed so that the distance between the heated film of the dinitrophenyl derivatives and the cooled glass surface (carbon dioxide snow + acetone in the cooling finger) is very short.

The ether extracts are placed in these special tubes and evaporated to dryness. If, after evaporation of the ether, a droplet of water remains in the tube, a glass cap (with an outlet tube) is placed on the ground glass point of the tube. The water is easily removed under reduced pressure in a few minutes. Sublimation of the dinitrophenol is effected at 70–80° (the tubes are not placed in the water bath until after a vacuum has been established) during 30 min*.

4. DINITROPHENYLATION OF A PROTEIN (Table 3)

The dinitrophenylation of a protein was first described by SANGER[76]. Many procedures can be employed.

(*a*) *Coupling technique with fluorodinitrobenzene*

Coupling may be effected with native, denatured or oxidized proteins.

With native proteins, a small number of ε-amino groups of intrapeptidic residues of lysine and of imidazole nuclei of histidine residues may not be able to react with fluorodinitrobenzene (PORTER[65]). This absence of reactivity in no way interferes with the determination of the N-terminal amino acid residues.

In the case of cystine or of a half-residue of cystine in N-terminal position (as in chymotrypsinogen; BETTELHEIM[10]), it is essential to effect the identification in the form of DNP-cysteic acid. For this the oxidized protein can be dinitrophenylated or the DNP-protein oxidized. The oxidation of the protein should be carried out very gently, notably by the procedures of THOMPSON[87] and HIRS[34]**.

1. *Coupling in an ethanol–bicarbonate medium* (SANGER[76])

The protein (0.5 g, for example) and sodium bicarbonate (0.5 g) are dissolved in 5 ml of water. To the solution are added 10 ml of a 5 % (v/v) ethanol solution of fluorodinitrobenzene, and the mixture is agitated mechanically for 2 to 3 h in the dark at room temperature.

The dinitrophenylation of an insoluble protein requires a prolonged period of agitation (48 h at 40°, 72 h at 20°) and repeated additions of sodium bicarbonate and fluorodinitrobenzene.***.

After complete dinitrophenylation, the DNP-protein is often insoluble, even in an alkaline medium; on the other hand, after acidification of the medium most of the DNP-proteins are precipitated. After centrifugation, the precipitate is washed several

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* During sublimation a small amount of DNP-methionine may be removed with the dinitrophenol.

** To avoid destruction of the DNP-amino acids, it is essential to eliminate the last trace of hydrogen peroxide. This can be done by precipitating the oxidized DNP-protein with peroxide-free ether followed by dissolution in formic acid and reprecipitation with ether, repeating this 3 times.

*** For the dinitrophenylation of wool at 60° see FRITZE AND ZAHN[29a].

times with water (to remove mineral salts), with alcohol until a colourless supernatant liquid is obtained (to eliminate the excess of fluorodinitrobenzene and the dinitrophenol formed) and finally with ether.

The washing may be modified in accordance with the particular solubilities of the DNP-proteins (DNP-salmine and DNP-ovomucoid are soluble in water and can be precipitated with excess of ethanol, the DNP-derivatives of acid glycoprotein α_1 of the serum or DNP-orosomucoid are soluble in water and ethanol). Elimination of the reagents or of the artefacts of condensation can always be achieved by dialysis, and the solution of DNP-protein can be lyophilized.

2. *Coupling in aqueous medium*

LEVY AND LI[47] have described a coupling reaction in aqueous medium maintained at pH 8 by means of an auto-titrator (JACOBSEN-LEONIS model, for example).

The protein (at least 0.2 mole) is dissolved in 3 ml of 0.1 *M* potassium chloride at 40°. The pH is adjusted and maintained at 8 by additions of 0.05 *N* KOH. After the addition of fluorodinitrobenzene (approx. 0.1 ml) the solution is agitated vigorously. The coupling reaction is terminated after agitation for 90 to 120 min: the curve of KOH consumption in relation to the time shows an inflexion when the reaction is terminated; the slope of the curve, which then becomes constant, corresponds to the hydrolysis of fluorodinitrobenzene.

The DNP-protein may remain soluble in the reaction medium, which is then extracted (3 times) with ether to remove the excess of fluorodinitrobenzene. After acidification, the DNP-protein is precipitated. It is collected by centrifugation, washed with water, acetone and ether, and dried over P_2O_5.

3. *Coupling in a bicarbonate–guanidine hydrochloride medium*

According to PHILLIPS[64], a more satisfactory yield of terminal groups is obtained by carrying out the dinitrophenylation in a solution of potassium bicarbonate and guanidine hydrochloride. The following procedure is recommended.

The protein is dissolved in a solution of 6 *M* guanidine hydrochloride (protein concentration 20 mg/ml). Solid potassium bicarbonate is added in a concentration of 10–15 mg/ml and fluorodinitrobenzene in a concentration of 0.05–0.1 ml/ml. The mixture is agitated at 20° for a time which may vary from 6 to 24 h. It is then acidified, diluted with three volumes of water and extracted once with ether, the two phases being readily separated by centrifugation.

The precipitate of DNP-protein is washed with water several times by centrifugation. The last traces of reagent and of dinitrophenol are eliminated by three washings with acetone and one washing with ether.

(*b*) *Determination of the quantity of protein in the DNP-protein*

In order to determine the number of molecules of amino acids in N-terminal position per molecule of protein, it is essential to know exactly the quantity of protein present

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in the DNP-protein. As a first approximation, 80 % of the weight of DNP-protein corresponds to the initial protein.

With the synthesis procedures described above (especially the coupling procedure in an ethanol–bicarbonate medium), it is possible to operate carefully and quantitatively and to take into account exclusively the weight of protein effective at the moment of coupling.

In the protein and in the DNP-protein obtained, it is much easier to determine a group or a residue that has not been modified by the coupling reaction: the amido group does not react with fluorodinitrobenzene and can be determined simply and precisely.

According to SANGER[76], the DNP-protein is hydrolysed with boiling 2 *N* hydrochloric acid for 4 h. After neutralization, the liberated ammonia is distilled off in a micro-kjeldahl apparatus at 95°, employing a borate buffer of pH 9.5.

According to THOMPSON[85], the DNP-protein is hydrolysed for 9 h at 105° in a sealed tube with 2 *N* hydrochloric acid. After neutralization with 2 *N* NaOH at pH 5 (bromocresol green), the ammonia is distilled off after addition of phosphate buffer 0.15 *M* of pH 10.5.

It should be noted that the molecular weight of the DNP-protein can be calculated when the total number of amino acid residues of the protein is known, assuming that the intrapeptidic residues of lysine, tyrosine, histidine, cysteine and all the residues in N-terminal position have been substituted. It is only necessary to add to the molecular weight of the protein the value obtained by multiplying the total number of dinitrophenyl radicals introduced by 166.

(*c*) *Hydrolysis of the DNP-protein*

1. *Total hydrolysis of the DNP-protein*

Conditions for the total hydrolysis of the protein, in view of the determination of the amino acid residue in the N-terminal position, vary according to the substrates and according to the nature of the terminal amino acid. It is necessary that the hydrolysis should be carried to such a point that the hydrolysate contains no (or little) DNP-peptides, and that the time of hydrolysis is not too long, in order to avoid as far as possible destruction of the terminal DNP-amino acid (for the rate of destruction of the DNP-amino acids during the course of hydrolysis, see: Quantitative chromatography, Section 10, b, p. 102).

For the qualitative determination of the N-terminal residue, hydrolysis is generally effected in a sealed evacuated tube (or filled with nitrogen) for 16 h at 105° in the presence of redistilled 5.7 *N* hydrochloric acid (see: Hydrolysis of the protein, Section 3, a, p. 66).

This type of hydrolysis, however, may cause considerable destruction of DNP-proline and of DNP-glycine.

Hydrolysis of DNP-proline for 16 h with 12 *N* hydrochloric acid involves the destruction of more than 50 %*. During hydrolysis rupture of the ring of DNP-proline

* Hydrolysis with 96% acetic acid for 16 h at 100° also destroys 50% of the DNP-proline. Under these conditions the DNP-protein is not completely hydrolysed (SCANES AND TOZER[81]).

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may give rise to two derivatives, α-chloro-δ-DNP-aminovaleric acid and δ-chloro-α-DNP-aminovaleric acid (see Section 7, a, 5, p. 86).

After 8 h of hydrolysis with 5.7 *N* hydrochloric acid 60 % of DNP-glycine is destroyed (PORTER[66]).

To avoid this destruction as far as possible it is recommended to carry out, in addition to hydrolysis at 105° for 16 h, two other hydrolyses:

For DNP-glycine, hydrolysis with 5.7 *N* hydrochloric acid for 4 h; under these conditions it is essential to determine whether the hydrolysate contains DNP-peptides.

For DNP-proline, hydrolysis for 4 h with 11.2 *N* hydrochloric acid (PORTER AND SANGER[67]; PHILLIPS[64]). SHEPHERD *et al.*[83] recommend also a hydrolysis for 24 h at 105° with a mixture of equal parts of acetic acid and hydrochloric acid.

Destruction of the DNP-amino acids during acid hydrolysis is very definitely increased in the presence of tryptophan or proteins rich in tryptophan such as lysozyme (THOMPSON[84]). Xanthylation of the tryptophan or of the protein very definitely reduces the rate of destruction (DICKMAN AND ASPLUND[25]), dixanthyltryptophan being stable to acid hydrolysis. It is therefore sometimes useful to xanthylate the DNP-protein. The DNP-protein is dissolved in 90 % acetic acid in the presence of xanthydrol. After an hour of contact at room temperature the xanthyl-DNP-protein is precipitated with ether and washed with ether by centrifugation.

2. *Partial acid hydrolysis of the DNP-protein*

It is sometimes useful to hydrolyse the DNP-protein partially in such a way that the DNP-peptides of the N-terminal sequence can be isolated and their structure determined. This partial hydrolysis can be achieved by the action of 12 *N* hydrochloric acid at 37° or of 5.6 *N*, 3 *N* or 0.1 *N* hydrochloric acid at 100°, during different lengths of time.

3. *Enzymic hydrolysis of the DNP-protein*

The proteolytic enzymes can partially hydrolyse certain DNP-proteins. Usually the velocity of the reaction is definitely small and the degree of hydrolysis low. Enzymic hydrolysis also makes it possible to tackle the problem of the structure of the N-terminal DNP-peptides. Interesting examples have been described: for example, peptic hydrolysis of DNP-ribonuclease (ANFINSEN *et al.*[2]), specific tryptic hydrolysis of oxidized DNP-ribonuclease (REDFIELD AND ANFINSEN[70]), hydrolysis of a DNP-protein by carboxypeptidase with the object of determining the C-terminal sequence (WALDSCHMIDT-LEITZ AND GAUSS[90]).

(*d*) *Extraction of dinitrophenylamino acids*

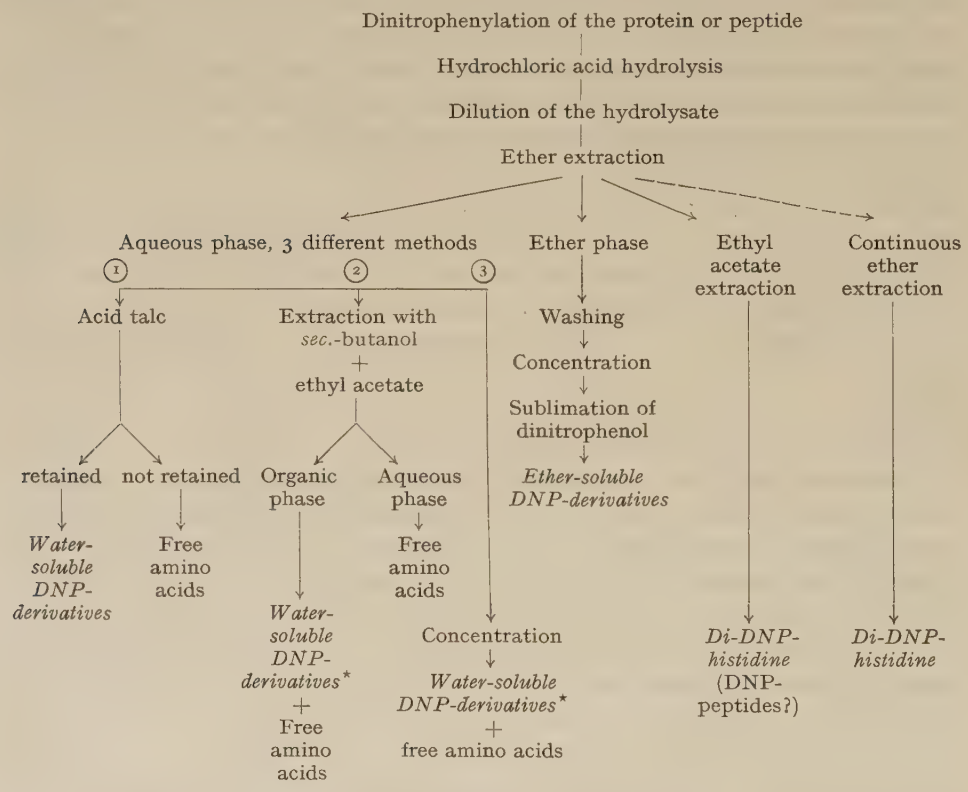
1. *Extraction from a total hydrolysate of proteins*

After hydrolysis, the hydrolysates are diluted with water until the concentration of hydrochloric acid is approximately normal. The diluted hydrolysate is extracted 4 times with 5 ml of peroxide-free ether (see footnote, p. 67). The ether extracts are

washed with 0.1 *N* hydrochloric acid (PHILLIPS[64]) or 3 times with water (THOMPSON[85]) to eliminate all traces of acid-soluble derivatives (such as ε-mono-DNP-lysine) that may have been carried over in the course of extraction. The ether extracts are concentrated and the dinitrophenol with which they are contaminated is eliminated by sublimation according to the methods described above (Section 3, c, p. 69). The ether-soluble DNP-amino acids are dissolved in a known volume of acetone. An aliquot part of this solution is placed on sheets of Whatman No. 1 paper. The ether-soluble DNP-amino acids may contain traces of di-DNP-histidine when this is in the N-terminal position. Continuous extraction with ether in a special apparatus (MILLS[58]) makes it possible to obtain this derivative also in the ether-soluble fraction. Extrac-

TABLE 3

DETERMINATION OF THE AMINO ACID WITH A FREE TERMINAL α-AMINO GROUP (SANGER'S method[76])



* The water-soluble DNP-derivatives consist of:

(1) *Derivatives from non-terminal amino acids*: ε-mono-DNP-lysine, imidazole-DNP-histidine (colourless), O-DNP-tyrosine (colourless), S-DNP-cysteine (colourless); sometimes δ-mono-DNP-ornithine and γ-mono-DNP-diaminobutyric acid (bacterial peptides).

(2) *Derivatives from terminal amino acids*: α-mono-DNP-arginine, di-DNP-histidine, α-mono-DNP-histidine, DNP-cysteic acid; in special cases: α-mono-DNP-lysine, α-mono-DNP-ornithine, α-mono-DNP-diaminobutyric acid.

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tion with ethyl acetate, after the extraction with ether, also leads to the isolation of this compound, but it must be borne in mind that any DNP-peptides present may also be extracted by this solvent (see Section 4, d, 2, below).

The residual aqueous phase of the hydrolysate contains all the free amino acids, always the water-soluble DNP-amino acids such as ε-mono-DNP-lysine, O-DNP-tyrosine (colourless), imidazole-DNP-histidine, and sometimes S-DNP-cysteine, which are derived from the corresponding residues of intrapeptidic amino acids. One may also find α-mono-DNP-arginine, DNP-cysteic acid (oxidized hydrolysate of protein or hydrolysate of oxidized protein), di-DNP-histidine (if the hydrolysate has not been subjected to continuous extraction with ether or to successive extraction with ether and ethyl acetate) and α-mono-DNP-histidine, when the corresponding amino acids are present in the protein in N-terminal position.

The water-soluble dinitrophenyl derivatives can be extracted (although this is not necessary) with *sec.*-butanol. The organic phases thus obtained and the remaining aqueous phase are evaporated to dryness, taken up in a known volume of acetone (slightly acid if necessary) and studied by partition chromatography on paper. Separation of the dinitrophenyl derivatives from the aqueous phase and from the free amino acids (all the constituent amino acids plus a few "non-reactive" molecules of lysine and histidine) can be achieved on a column of talc (SANGER[78]). The aqueous residue is dissolved in 2 ml of *N* hydrochloric acid and passed through a column (2.5 cm diameter) containing a mixture of 20 g Hyflo-Super-Cel and 50 g of talc* moistened with *N* hydrochloric acid. The free amino acids are not adsorbed whereas all the dinitrophenyl derivatives (except that of cysteic acid: REDFIELD AND ANFINSEN[70]) are adsorbed. After careful washing of the column with hydrochloric acid (100 ml), the dinitrophenyl derivatives are eluted with 400 ml hydrochloric alcohol (alcohol 4 vol., *N* hydrochloric acid 1 vol.). The elution can also be carried out with 80 % ethanol containing 0.3 % of ammonia (BAILEY AND BETTELHEIM[5]). The eluate can be evaporated. If the residue is treated with fluorodinitrobenzene ε-DNP-lysine is converted to di-DNP-lysine, O-DNP-tyrosine to di-DNP-tyrosine, and imidazole-DNP-histidine to di-DNP-histidine, whereas α-mono-DNP-arginine remains unchanged. After this second dinitrophenylation, ether extraction of the residue removes di-DNP-lysine and di-DNP-tyrosine, ethyl acetate extraction can remove di-DNP-histidine, while α-mono-DNP-arginine remains in the aqueous phase (see ROVERY, FABRE AND DESNUELLE[75]).

2. *Extraction from a partial hydrolysate of protein*

The extraction procedures are identical with those used for the total hydrolysates. By successive extractions with ether, ethyl acetate and *n*-butanol (WOOLLEY[94], SANGER[78]) a certain basic fractionation of the mixture can be achieved.

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* It is essential to remove the fine particles of talc which interfere with the flow. By boiling the suspension of talc in 0.01 *N* hydrochloric acid the fine particles are collected on the surface of the liquid and can be readily removed. Washing is repeated several times (BAILEY AND BETTELHEIM[5]).

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It is very important to be able to separate as completely as possible the α-DNP-peptides of the N-terminal sequence, the acid form of which is in principle soluble in the organic solvents, from the non-terminal peptides of a yellow colour and containing ε-DNP-lysine, which are in principle retained in the aqueous phase because of their free amino groups. Unfortunately such a separation is not always very sharp (DESNUELLE AND FABRE[23]; SCHROEDER[82]).

The ethyl acetate extracts may contain ε-mono-DNP-lysine. It is useful to wash the various organic phases several times with water and determine the composition of these washing liquids.

Peptides containing ε-DNP-lysine can be separated from the other unsubstituted peptides and from the free amino acids on a column (1 cm in diameter) containing 5 g of talc washed with *N* hydrochloric acid (SANGER[78]). Peptides containing ε-DNP-lysine are adsorbed on the column. After washing with *N* hydrochloric acid (40 ml) they are eluted with a mixture of ethanol (4 parts) and hydrochloric acid (1 part) or, preferably, with 80 % ethanol containing 0.3 % of ammonia.

5. DINITROPHENYLATION OF A PEPTIDE

In practice the dinitrophenylation of a peptide may be carried out in exactly the same way as that of a protein. However, certain methods (particularly micro-methods) are especially suitable for this coupling reaction. The amounts of peptides employed are generally very small.

1. *Dinitrophenylation in a trimethylamine medium* (SANGER AND THOMPSON[79])

Substitution of trimethylamine for sodium bicarbonate results in a diminution of the ionisation of the medium; moreover this reagent can be conveniently eliminated afterwards. The peptide (0.2 μmole for example) is dissolved in 0.1 ml of 1 % trimethylamine. A solution of 10 μl of fluorodinitrobenzene in 0.2 ml of ethanol is added. After 2 h contact a few drops of water and of the trimethylamine solution are added and the excess of fluorodinitrobenzene is removed by extracting 3 times with ether or with ether containing 1 % of triethylamine (HAUSMANN *et al.*[32]). After evaporating the aqueous solution to dryness, the residue is hydrolysed directly with 5.7 *N* hydrochloric acid.

2. *Dinitrophenylation in a trimethylamine carbonate medium*

To reduce the formation of dinitrophenol, LOCKHART AND ABRAHAM[50] replace trimethylamine by trimethylamine carbonate and proceed as follows:

The peptide (50–150 μg) is dissolved in 0.1 ml of a 1.5 % solution of trimethylamine carbonate (w/v) (pH 9.3), 0.01 ml of fluorodinitrobenzene in 0.2 ml of ethanol is added and the reaction is allowed to take place in the dark during 2 h 30 min. The ethanol is expelled under reduced pressure and 0.24 ml of trimethylamine carbonate solution is added. The excess of fluorodinitrobenzene is removed by extraction with ether and the aqueous solution is evaporated to dryness *in vacuo*. The residue, dissolved in 0.1 ml of 6 *N* hydrochloric acid, is hydrolysed for 9 h at 105° in a sealed

tube under nitrogen. The hydrolysate is diluted with 2 volumes of water and the ether-soluble DNP-amino acids are extracted 3 times with an equal volume of ether. Any di-DNP-histidine present can be extracted with *n*-butanol or ethyl acetate.

WALEY[91] recommends a method somewhat similar to the foregoing with a buffer of trimethylamine carbonate obtained by treatment of a 6% (v/v) solution of trimethylamine with carbon dioxide until alkaline to phenol red, but neutral to phenolphthalein. Under these conditions the dinitrophenylation is conducted at a slightly lower pH than in the method of LOCKHART AND ABRAHAM. A small amount of dinitrophenol is formed.

6. PAPER CHROMATOGRAPHY OF ETHER-SOLUBLE DINITROPHENYL DERIVATIVES

Separation of all the ether-soluble dinitrophenyl derivatives should be effected by means of two-dimensional chromatography.

Three types of chromatogram can be employed. In exceptional cases, with very simple mixtures, one (or several) one-dimensional chromatograms may suffice, provided that reference substances are run on the same sheet.

(*a*) *"Toluene" and "phosphate" solvents* (BISERTE AND OSTEUX[11]; LEVY[43])

The vessel used for the first ascending dimension of the chromatogram consists of a glass receptacle, either cylindrical or of rectangular cross section (height: 46 cm, cross section 23 × 23 cm), which must be hermetically sealed by a very even glass plate. The air-tightness of the vessel is ensured by means of silicon grease applied to the rim of the vessel or better by placing soft rubber between the edges of the vessel and the lid. A weight ensures that this lid is kept in place. At the bottom of the vessel two crystallizing dishes with a perfectly flat bottom (diameters 19 cm and 15 cm) are placed, one inside the other, leaving a substantial annular space in between. The vessel is placed in darkness in a room at constant temperature (21° ± 0.5°).

The first dimension of the chromatogram is realised with the "toluene" system of BISERTE AND OSTEUX[11], modified by LEVY[43], the composition of which is as follows: toluene–pyridine–ethylene chlorohydrin(2-chloroethanol)–ammonia 0.8 *N* (30:9:18:18). The reagents must be very carefully purified*.

The "toluene" solvent (two phases) is equilibrated, after shaking the separating funnel for once, at least 3 to 4 h. The lower aqueous phase is rejected and the organic phase is filtered through Whatman No. 1 paper to remove the few droplets of water that remain.

The dinitrophenyl derivatives in acetone solution are deposited on the corner of

* *Toluene*: extraction with pure sulphuric acid (100 ml of acid per l of toluene) during 24 h; after decantation, distillation over aluminium chloride, washing with 20% sodium carbonate, several washes with water (5 times), filtration on a filter containing pieces of paper which serve to bind the water, storage over dry calcium chloride and redistillation. *Chloroethanol*: distillation. *Pyridine*: distillation over baryta and redistillation.

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a sheet of Whatman No. 1 paper (40 × 55 cm) (see Fig. 4) by means of a micropipette. After application of the DNP-amino acids, the sheet of paper is rolled into a cylinder and fastened with clasps, which keep it in shape.

The roll is placed in the annular space between the two crystallizing dishes. 200 ml of 0.8 *N* ammonia (57 ml of concentrated ammonia 22° Bé, density = 0.925, in 1 l of water) are put into the central dish and 30 to 40 ml of the organic phase of the "toluene" solvent, prepared as indicated above, are placed at the bottom of the vessel outside the dishes. The chromatography vessel is very carefully sealed and the paper is allowed to reach equilibrium with the atmosphere of the vessel for at least 5 to 6 h. Prolongation of this period of equilibration (24 h for example) further

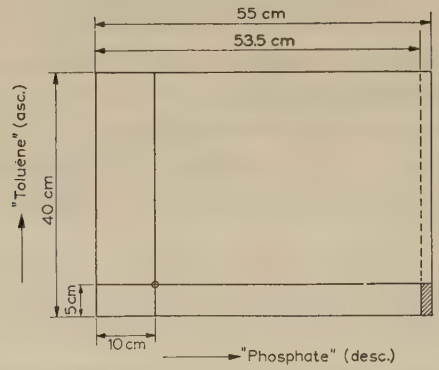

Fig. 4. Arrangement of the chromatogram (Whatman No. 1 paper). desc. = descending chromatography; asc. = ascending chromatography. After rolling the sheet of paper into a cylinder and fastening the two edges, it is advisable to cut off a piece of the paper (hatched surface in the figure; 5 × 1.5 cm) in order to avoid a too rapid mounting of the "toluene" solvent by capillarity.

improves the separation. It should be pointed out that the most satisfactory chromatograms are obtained in vessels that are continually in use.

At the end of this time a sufficient quantity (50 ml) of the organic phase of the "toluene" solvent is poured rapidly into the annular space, using a funnel with a long stem. The duration of the ascending development is 15 h. The next day the sheets are removed, dried in a current of warm air for 10 to 12 h *protected from light* under a hood. The removal of the first solvent must be complete.

The sheet is then unfolded, turned and placed in a tightly closing glass vessel (dimensions: height 48 cm, cross section 46 × 36 cm), for descending chromatography with a solvent system consisting of a 1.5 *M* phosphate buffer of pH 6: 1 *M* NaH_2PO_4, 0.5 *M* Na_2HPO_4, *i.e.* 138 g of $NaH_2PO_4 \cdot H_2O$ and 71 g of Na_2HPO_4 per litre, or 156 g of $NaH_2PO_4 \cdot 2H_2O$ and 89 g of $Na_2HPO_4 \cdot 2H_2O$ per litre*.

The development may be started immediately without a period of equilibration. It is necessary to place a little cup filled with water on the bottom of the vessel.

* It is recommended to prepare a buffer of higher molarity: 3 *M* (2 *M* NaH_2PO_4 1 *M* Na_2HPO_4, *i.e.* 312 g of $NaH_2PO_4 \cdot 2H_2O$ and 178 g of $Na_2HPO_4 \cdot 2H_2O$ per litre). This solution can be diluted immediately before making the chromatogram. It can be kept in an oven at 37°.

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The duration of the development, which is carried out in the dark in a room at constant temperature (+ 21° ± 0.5°) is 15 h. The chromatogram is dried for 6 h at least in a current of warm air, protected from light, under a hood.

This type of two-dimensional chromatography permits the separation of most of the ether-soluble DNP-amino acids, with the exception of dicarboxylic DNP-amino

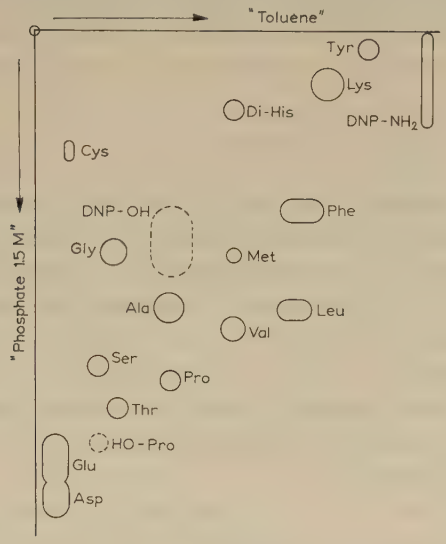

Fig. 5. Chromatographic map of ether-soluble DNP-amino acids from a total hydrolysate of protein. Solvent systems: "toluene" and 1.5 *M* phosphate. DNP-NH_2 = dinitraniline; DNP-OH = dinitrophenol. On the diagram the dinitrophenylamino acids are indicated as the corresponding amino acids, *e.g.*: Ala = DNP-alanine. HO-Pro = DNP-hydroxyproline. Di-His = di-DNP-histidine.

acids (DNP-aspartic acid, DNP-glutamic acid). These two derivatives can be separated by means of a second chromatogram, using the "toluene" system in the first dimension and a phosphate buffer of higher concentration (2.5 *M*) in the second dimension, the descending development being prolonged for 48 h. The separation obtained in the case of a mixture of ether-soluble DNP-amino acids from a total hydrolysate of protein is shown diagrammatically in Fig. 5.

It is not necessary to define the position of each spot by the R_F in the two dimensions. In fact the relative position of each derivative is always very characteristic; moreover the R_F values, notably in the "toluene" solvent, may vary within certain limits according to the type of the chromatography vessel. Of the other causes of variations in the R_F value, the following may also be mentioned: the degree of saturation of the atmosphere in the vessel, the ageing of the "toluene" solvent, the distance travelled by the front of the solvent, the composition of the mixture and the quantity of the DNP-amino acids. In the case of simple mixtures, from which many DNP-amino acids are absent, exact identification can be attained easily and with certainty by using internal reference substances. Placed on the starting point, at the same time as the mixture being studied, these reference substances fix the positions of the spots for

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identification. The traces of dinitrophenol which may still be present in the mixtures being studied and which are easily and completely decolorized on coming into contact with vapours of formic acid also serve as indicator for the identification of DNP-amino acids. The indication and the localization of small quantities of DNP-amino acids are facilitated by a *rapid* examination in Wood's light (brown fluorescence); 2,4-dinitroaniline, which is a constant artefact of the method, exhibits under these conditions a very specific green-yellow fluorescence. Moreover it has an R_F value in the vicinity of 1 in the "toluene" system and a very low one in the phosphate buffer.

(*b*) *n-Butanol–NH_3 and "phosphate" solvents*

BRAUNITZER[15] has recommended the use of the system *n*-butanol saturated with 0.1 % ammonia (w/v) (see also KOCH AND WEIDEL[39]; DAVIES AND HARRIS[22]) in the first ascending dimension. The system propanol–0.2 % ammonia (w/v) (75:25) can also be employed. It is preferable here to chromatograph on Schleicher & Schüll paper No. 2043a or 2043b.

The second dimension is developed either with the 1.5 *M* phosphate buffer (pH 6) of LEVY (KOCH AND WEIDEL[39]) or with more dilute phosphate buffer, 0.75 *M* of pH 6 (DAVIES AND HARRIES[22]). The duration of the development with the 0.75 *M* phosphate is reduced to 7–8 h.

The experimental methods for the development are in all points comparable to those that we have described for "toluene" and "phosphate" chromatography. The position of the ether-soluble DNP-amino acids is shown diagrammatically in Fig. 6.

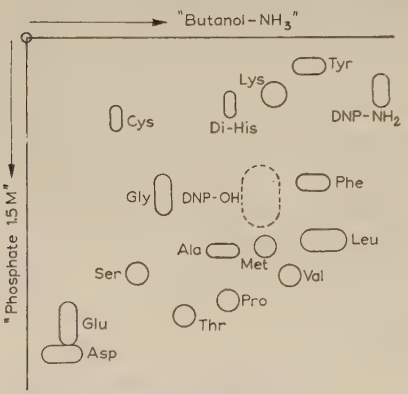

Fig. 6. Chromatographic map of the ether-soluble DNP-amino acids from a total hydrolysate of protein. Solvent systems: butanol–NH_3 and 1.5 *M* phosphate. DNP-NH_2 = dinitraniline; DNP-OH = dinitrophenol; Di-His = di-DNP-histidine.

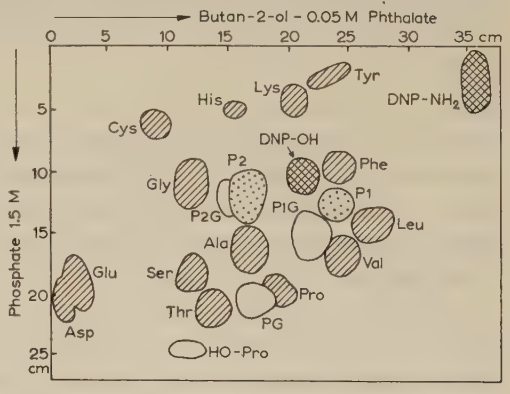

Fig. 7. Chromatographic map of the ether-soluble DNP-amino acids from a total hydrolysate of protein. Solvent systems: *sec.*-butanol–pH 6 phthalate and 1.5 *M* phosphate (according to PHILLIPS[64]). DNP-OH = dinitrophenol; DNP-NH_2 = dinitraniline; P1 = δ-chloro-α-DNP-aminovaleric acid; P2 = α-chloro-δ-DNP-aminovaleric acid; PG = DNP-Prolylglycine; P1G = δ-choro-α-valerylglycine; P2G = probably α-chloro-δ-DNP-aminovalerylglycine. The ether-soluble DNP-amino acids usually found in a hydrolysate are indicated by the hatched surfaces, the products of decomposition of DNP-proline by the dotted surfaces. ε-DNP-lysine (water-soluble) coincides with DNP-serine.

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(c) *sec.-Butanol–pH 6 phthalate and "phosphate" solvents*

PHILLIPS[64] has described a two-dimensional chromatography on Whatman No. 7 paper in which the solvent butan-2-ol saturated with 0.05 *M* phthalate buffer of pH 6* is used in the first dimension (duration of development: 15 to 20 h). The paper is previously impregnated with the 0.05 *M* phthalate buffer. LEVY's system of 1.5 *M* phosphate buffer of pH 6 is used in the second dimension (duration of development: one night) (Fig. 7).

(d) *tert.-Amyl alcohol–pH 6 phthalate and 1.5 M phosphate solvents*

PHILLIPS[64] has also suggested a two-dimensional procedure, in which the system of BLACKBURN AND LOWTHER[13] (*tert*-amyl alcohol—or 2-methylbutan-2-ol—saturated with 0.05 *M* phthalate buffer of pH 6*; time of development in the dark: 30–40 h) is used in the first dimension. Prolonged equilibration (one night) of the paper before commencing chromatography improves the results (INGRAM AND SALTON[36]). The second dimension is achieved with the 1.5 *M* phosphate buffer of pH 6 of LEVY, after drying at room temperature.

The sheets of Whatman No. 7 paper are impregnated with the 0.05 *M* phthalate buffer of pH 6 previous to development with the first solvent. Whatman No. 4 or 3 MM paper is sometimes used with the solvent system of BLACKBURN AND LOWTHER (LOCKHART, ABRAHAM AND NEWTON[51]).

(e) *Comparison of different techniques*

There are essential differences between these various techniques owing to the nature and composition of the solvent system used in the first dimension. It is interesting to note that the general distribution and the relative positions of the dinitrophenyl derivatives are always substantially the same, whichever solvent system is employed in the first dimension. Therefore the separation obtained does not seem to be due solely to partition. On the other hand chromatography in the second dimension is essentially a salting-out effect.

According to the combinations used, the position of dinitrophenol, which is the principal artefact in SANGER's method[76], may vary considerably. With the combination "toluene + phosphate", dinitrophenol is close to DNP-glycine and DNP-alanine. With the combinations *n*-butanol–NH_3 + phosphate and *sec.*-butanol–phthalate pH 6 + phosphate, dinitrophenol is much closer to the DNP-phenylalanine, DNP-leucine, DNP-valine, DNP-methionine group, which position is much less convenient. These difficulties are of course less if the dinitrophenol has been eliminated by sublimation.

Nevertheless, on the whole we consider that the most satisfactory resolution is obtained with the system "toluene + 1.5 *M* phosphate". The use of a 0.75 *M* phosphate

* Formula of the phthalate buffer: 50 ml of 0.1 *M* potassium hydrogen phthalate (20.418 g per l) and 45.45 ml of 0.1 *N* NaOH; dilute to 100 ml with water free from CO_2.

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buffer makes it possible to reduce the time of development of the second dimension. As PHILLIPS[64] points out, it is inconvenient to employ the system toluene–pyridine–chloroethanol–ammonia in laboratories where determinations of amino acids are made by colorimetric methods using ninhydrin.

(*f*) *Other solvent systems*

All the problems encountered in the separation of the ether-soluble DNP-amino acids can be solved by the two-dimensional combinations described above. A few simple problems can be solved using one of the above-mentioned solvent systems in one-dimensional chromatography, provided that reference substances are placed on the chromatogram (lateral indicators and internal indicators). However, other solvent systems have been proposed. But their use is confined to certain very special problems. Among the most useful we may mention:

Water–benzene–acetic acid (1:1:1) (PARTRIDGE AND DAVIS, quoted by SANGER AND THOMPSON[79]), suitable for the DNP-amino acids with low R_F values in organic solvents: DNP-aspartic acid, DNP-glutamic acid, DNP-serine and perhaps DNP-cysteic acid.

Solvent systems of MELLON *et al.*[55] (see Table 4):

Solvent A = *n*-butanol, water-saturated;

Solvent B = *n*-butanol–*n*-butyl acetate–1 % ammonia (v/v) (1:2:3) (prepared 18 h before use);

Solvent C = benzene–1 % acetic acid; ascending development on Whatman

TABLE 4

R_F VALUES OF 2,4-DINITROPHENYLAMINO ACIDS
(according to MELLON, KORN AND HOOVER[55])

Amino acids	*Solvent A**	*Solvent B*	*Solvent C*
Dinitroaniline	0.90	0.97	0.96
Di-DNP-tyrosine	0.78	0.90	0.33
Leucine	0.74	0.71	0.70
Isoleucine	0.73	0.70	0.70
Di-DNP-lysine	0.72	0.81	0.11
Phenylalanine	0.71	0.70	0.55
Tryptophan	0.70	0.68	0.28
Valine	0.68	0.47	0.63
Methionine	0.65	0.48	0.47
Dinitrophenol	0.56	0.25	0.99
Alanine	0.50	0.18	0.28
Proline	0.48	0.17	0.44
Threonine	0.43	0.12	0
Glycine	0.36	0.08	0.07
Serine	0.32	0.06	0
Glutamic acid	0.14	0	0
Aspartic acid	0.12	0	0
Di-DNP-histidine	0.35	0.50	0
Arginine	0.37	0	0
ε-DNP-lysine	0.32	0.05	0
α-DNP-lysine	0.33	0	0

* For the composition of the solvents, see text.

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No. 1 paper; the paper is equilibrated over-night before the organic solvent is added; the solvent system C gives streaks.

Isooctane–ethylene chlorohydrin–n-propanol (20:1:1) (WILLIAMSON AND PASSMANN[92]): separation of DNP-leucine and DNP-phenylalanine.

Decaline (decahydronaphthalene)–glacial acetic acid (1:1) (BISERTE AND OSTEUX[11]) suitable for the separation of dinitrophenol and DNP-amino acids.

n-Propanol (diluted with water to a density of 0.813)–acetic acid (containing 1.5% of water)–kerosene (b.p. 100–140°) (20:6:100), in descending chromatography on Whatman No. 1 paper soaked in 0.1 *M* citric acid and air-dried; suitable for the separation of the polymers of α-, β- and ω-amino acids (HEIKENS, HERMANS AND VAN VELDEN[33]).

Xylene–acetic acid–0.05 M phthalate buffer of pH 6 (10:5:4) (LANDMANN, DRAKE AND WHITE[42]); the paper is impregnated with the same buffer, then equilibrated with the lower layer 16 h before commencement of the development.

n-Butanol–ethanol–water (40:10:50) (v/v) (KENT, LAWSON AND SENIOR[38]).

Benzyl alcohol containing 10% ethanol (v/v), saturated with pH 6 0.05 M phthalate buffer (BLACKBURN AND LOWTHER[13]) (see Table 5).

Propanol–cyclohexane or petroleum ether (b.p. 100-120°) (30:70 v/v), saturated with pH 6 0.05 M phthalate buffer (BLACKBURN AND LOWTHER[13]) (see Table 5).

TABLE 5

R_F VALUES OF DNP-AMINO ACIDS ON BUFFERED PAPER (pH 6 PHTHALATE BUFFER)
(According to BLACKBURN AND LOWTHER[13])

	Cyclohexane containing 30% propanol	*tert.-amyl alcohol*	*Benzyl alcohol containing 10% ethanol*
DNP-leucine	0.28	0.88	0.71
DNP-valine	0.23	0.79	0.59
DNP-phenylalanine	0.22	0.74	0.63
DNP-alanine	0.15	0.46	0.36
DNP-glycine	0.10	0.23	0.26
DNP-threonine	0.07	0.36	0.26
DNP-serine	0.05	0.21	0.18
DNP-glutamic acid	0.05	0.04	0.07
DNP-aspartic acid	0.02	slow	0.03

pH 6.2 sodium citrate M or 0.7 M–hydrochloric acid buffer (ROVERY AND FABRE[74]; DESNUELLE AND FABRE[23]); duration: 16 h; this is in fact an application of salting-out chromatography, comparable to that effected with the pH 6 1.5 *M* phosphate buffer of LEVY.

Solvent of BLACKBURN AND LOWTHER *(modified): tert.-pentanol containing 10% (v/v) of propan-2-ol saturated with phthalate buffer* (GREGORY AND YOUNG, unpublished results, quoted by WALEY[91]).

Chloroform–1.5 N acetic acid–n-propanol (10:6:10) (SANGER AND THOMPSON[79]): reversed phase chromatography on silicone coated paper (KRITCHEVSKY AND

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TISELIUS[41]); the paper is suspended in the vessel saturated with vapours of the organic phase for 3 h; then development is carried out with the aqueous phase; di-DNP-tyrosine and di-DNP-lysine do not migrate, whereas the other DNP-amino acids migrate fairly rapidly. Owing to the variability of the R_F it is essential to chromatograph reference DNP-amino acids on the same sheet.

Chloroform–propan-2-ol–0.05 M-potassium benzoate (*45:49:6 v/v*) or *cyclohexane–propan-2-ol–0.05 M-potassium benzoate* (*60:36:4 v/v*) (MONIER AND PENASSE[60]).

n-Amyl alcohol agitated with an equal volume of 2 N ammonia solution; the aqueous phase is used for the saturation of the vessel (BOWES AND MOSS[14]).

tert.-Amyl alcohol–methyl ethyl ketone–potassium benzoate (*54:40:6*) (MONIER AND JUTISZ[59]).

7. SPECIAL APPLICATIONS OF CHROMATOGRAPHY OF ETHER-SOLUBLE DNP-AMINO ACIDS

(*a*) *Total hydrolysates of a DNP-protein and DNP-amino acids of a total hydrolysate*

All the problems may be approached with two-dimensional development, using the combinations of solvents described above. We nevertheless give preference to the "toluene" and 1.5 *M* phosphate systems. Some special cases are difficult to resolve. It is sometimes necessary to elute the spots of the DNP-amino acids from the chromatogram in order to be able to investigate them further with other solvent systems.

1. *Elution of spots of DNP-amino acids*

Elution of the paper is carried out in the following manner. The zones of paper containing the DNP-amino acids are cut off, placed in centrifuge tubes and eluted with 2 to 3 ml of 2 % sodium bicarbonate for 15 min at a temperature of 50–55°. After cooling and acidification with dilute hydrochloric acid, the DNP-amino acids are extracted with peroxide-free ether. The ethereal solution is extracted with 2 to 3 ml of 2 % sodium bicarbonate in order to eliminate all traces of the solvent employed in the first development; after acidification, the second bicarbonate solution is extracted with ether. This ethereal solution (at most 2 to 3 ml) can be desiccated with anhydrous sodium sulphate. After evaporation or concentration the DNP-amino acids are deposited on a sheet of Whatman No. 1 paper and submitted to one-dimensional chromatography in the new solvent system.

2. *Separation of the DNP-dicarboxylic acids*

Only DNP-aspartic and DNP-glutamic acid are not separated by two-dimensional development. This problem can be solved by a second two-dimensional development in which the second dimension is realised with a phosphate buffer of higher concentration (2.5 *M*); this time the first dimension is preferably carried out with the butanol–(NH_3) system, which can be more rapidly evaporated than the "toluene" system.

The spots of the dicarboxylic DNP-amino acids can also be eluted from the two-

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dimensional chromatogram and the eluate subjected to one-dimensional chromatography with the isoamyl alcohol system saturated with 1% acetic acid (R_F of DNP-aspartic acid 0.30; R_F of DNP-glutamic acid 0.46) (BISERTE AND OSTEUX[11]).

3. *Superpositions of spots of DNP-amino acids*

(*i*) *Superposition of DNP-histidine and DNP-tryptophan.* In the two-dimensional development using "toluene + phosphate", DNP-histidine and DNP-tryptophan appear in the same position. However, this sets no special problems*, for on the one hand DNP-tryptophan is destroyed during acid hydrolysis, and on the other di-DNP-histidine does not accompany the ether-soluble DNP-amino acids except in the case of a combined ether + ethyl acetate extraction.

(*ii*) *Superposition of DNP-serine- and DNP-methionine sulphone.* It the system "toluene"–1.5 *M* phosphate, DNP-serine and DNP-methionine sulphone are superposed. Actually, the formation of DNP-methionine sulphone at the expense of DNP-methionine occurs only when the DNP-amino acids have been extracted with ether that has not been freed from peroxides. If the presence of DNP-methionine is suspected, the zone DNP-serine + DNP-threonine can be chromatographed again in the system *tert.*-amyl alcohol–phthalate of BLACKBURN AND LOWTHER[13].

(*iii*) *Dinitrophenyl derivatives of diamino acids.* Di-DNP-lysine, di-DNP-ornithine and di-DNP-diaminobutyric acid do not separate in "toluene"+1.5 *M* phosphate. A partial separation of di-DNP-lysine and di-DNP-ornithine can be achieved by chromatography with the system butanol–acetic acid–water (4:1:5).

The identification of these compounds is therefore difficult. To solve this problem (which may occasionally arise in the case of bacterial polypeptides), it is therefore essential to regenerate the constituent amino acids (see below, Section 7, a, 4) and identify them, for example by paper electrophoresis in a Durrum type apparatus at pH 3.9 (buffer: pyridine–acetic acid–water (30:100:4870)) for the separation of α,γ-diaminobutyric acid from the group ornithine + lysine, or at pH 11.7 (*N* ammonia) for the separation of lysine and ornithine.

4. *Problem of the DNP-leucines*

No solvent system yields a satisfactory separation of the DNP-leucines. When determining a residue in N-terminal position, the only means of solving this problem consists in regenerating the amino acid from its dinitrophenyl derivative.

Regeneration** can be brought about by heating in a sealed tube at 105° for 1 h with 0.3 *N* baryta (MILLS[58]). The baryta is then removed in the form of barium carbonate by passing a stream of carbon dioxide into the solution. After evaporation to dryness, the residue is analysed by paper chromatography.

* Separation of DNP-tryptophan and di-DNP-histidine can be effected in the systems of MELLON: benzene–1% acetic acid; water-saturated *n*-butanol (see Table 4), and in the system *sec.*-butanol–pH 6 phthalate buffer (R_F of DNP-Try = 0.54; R_F of DNP-His = 0.33). Duration of development: 40 h (personal results).

** These methods of regeneration are applicable for all the DNP-amino acids; they can only be used for identification, for the yields of recovered amino acids are small.

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The DNP-amino acids can also be heated in the presence of ammonia (density 0.880) in sealed tubes at 100° (LOWTHER[52]).

Chromatographic separation of leucine and isoleucine can be effected either by the system *tert.*-pentanol–water (WORK[95]), or by HÖGSTRÖM'S system[35], or by the mixture *n*-butanol–benzyl alcohol–water (CONSDEN *et al.*[20]).

5. *Decomposition products of DNP-proline*

When DNP-proline is hydrolysed, two new derivatives may be formed: δ-chloro-α-DNP-aminovaleric acid and α-chloro-δ-DNP-aminovaleric acid (SCANES AND TOZER[81])*. The decomposition of DNP-proline and the formation of these compounds depend

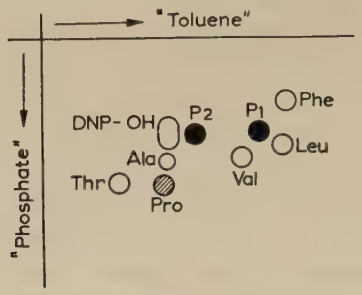

Fig. 8. Chromatographic separation (solvent systems: "toluene" and "phosphate") of the decomposition products of DNP-proline (hydrolysis with 5.6 *N* hydrochloric acid at 100–105° for 24 h in a sealed tube). DNP-proline = hatched surface; P1 = δ-chloro-α-DNP-aminovaleric acid; P2 = α-chloro-δ-DNP-aminovaleric acid; DNP-OH = dinitrophenol.

on the conditions of hydrolysis (see Section 4, c, 1, p. 72). It is convenient to identify DNP-proline in N-terminal position by means of these two artefacts (PHILLIPS[64]). Their chromatographic separation either by the "toluene" and "phosphate" solvents (see Fig. 8) or by the *sec.*-butanol–phthalate and "phosphate" solvents (see Fig. 7) is comparatively easy. It is interesting to note that DNP-proline shows a red coloration after prolonged exposure of the chromatogram to Wood's light.

6. *Chromatographic artefacts in the ether-soluble fraction*

Dinitrophenol and dinitraniline are the usual artefacts in the ether-soluble fraction. Dinitrophenol can be readily eliminated by sublimation** (see Section 3, c, p. 69). Dinitraniline does not interfere with the chromatographic separation, for it moves far from all the other dinitrophenyl derivatives.

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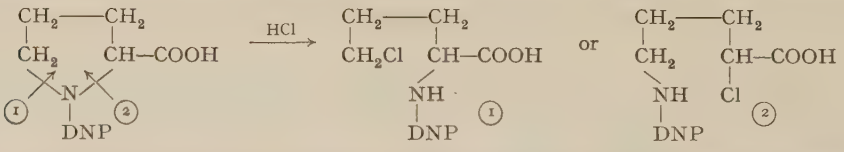
* The action of the hydrolysing agent could open the pyrrolidine ring according to the reaction:



** It can also be removed by means of a column of silicic acid Mallinckrodt, prepared for chromatography by the method of RAMSEY AND PATTERSON (LI AND ASH[48]).

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With certain preparations of fluorodinitrobenzene we have sometimes observed the presence of picric acid in a region close to that occupied by phenylalanine (see Fig. 9). The eluate of the spot gives the characteristic and classical reactions of picric acid.

Other artefacts have also been mentioned, notably by REDFIELD AND ANFINSEN[70]: a spot slower than phenylalanine in the "toluene" solvent and a little slower than leucine in the "phosphate" solvent; an orange-coloured artefact a little more rapid than glycine; another orange-coloured artefact in the region of valine. Finally, two decomposition products of ε-DNP-lysine (one yellow, the other orange) may appear in the zone comprised between di-DNP-lysine and di-DNP-histidine. These may be confused with di-DNP-histidine.

(b) *Enzymic hydrolysates of proteins; separation of DNP-asparagine and DNP-glutamine*

The only special problem encountered in the investigation of the DNP-amino acids from enzymic hydrolysates of proteins (obtained, for example, by the action of carboxypeptidase or of leucine-aminopeptidase) is that of the separation of DNP-asparagine and of DNP-glutamine.

In the solvents "toluene" and 1.5 *M* phosphate, these two derivatives in fact form only one spot, clearly separated from the other dinitrophenyl derivatives between DNP-glycine and the group DNP-serine–DNP-threonine (Fig. 9). After elution of this zone, as described above (Section 7, a, 1, p. 84), DNP-glutamine and

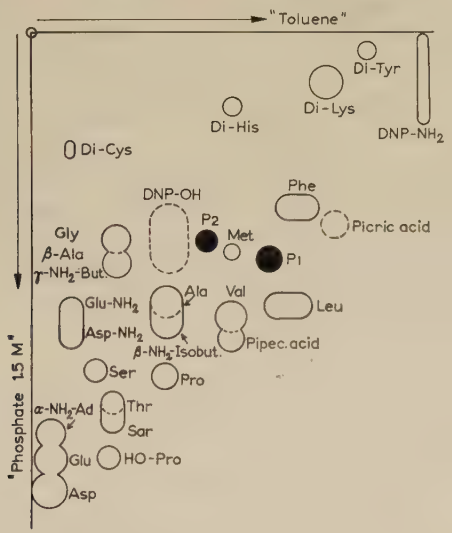

Fig. 9. Chromatography of ether-soluble DNP-amino acids detectable after dinitrophenylation of a biological medium. Solvent systems: toluene and 1.5 *M* phosphate. α-NH_2-Ad. = α-aminoadipic aicd; Sar = sarcosine; β-NH_2Isobut. = β-aminoisobutyric acid (T-spot); Pipec. acid = pipecolic acid; Asp.-NH_2 = asparagine; Glu-NH_2 = glutamine; β-Ala = β-alanine; γ-NH_2-But. = γ-aminobutyric acid; P 1 and P 2 = decomposition products of proline (see Fig. 8).

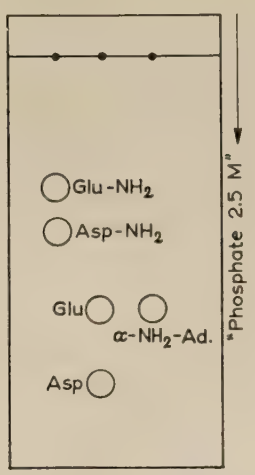

Fig. 10. Separation of dicarboxylic DNP-amino acids and their amides by means of the solvent system 2.5 *M* phosphate. Glu-NH_2 = glutamine; Asp-NH_2 = asparagine; α-NH_2-Ad. = α-amino-adipic acid.

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DNP-asparagine are readily separated by one-dimensional chromatography in a 2.5 *M* phosphate buffer (Fig. 10). DNP-Glutamine appears behind DNP-asparagine.

(*c*) *Complex biological media*

Dinitrophenylation of the amino acid fraction of a complex biological medium (blood, urine, tissues) leads to the formation of many new ether-soluble dinitrophenyl derivatives, which appear most often at the same level as the usual DNP-amino acids on the chromatograms obtained with the "toluene"–1.5 *M* phosphate system (Fig. 9).

α-DNP-aminoadipic acid cannot be separated from DNP-glutamic acid, even with a pH6 2.5 *M* phosphate buffer (Fig. 10).

DNP-sarcosine appears at the level of DNP-threonine. After elution of their common spot, these two derivatives can be distinguished from each other in the system butanol–acetic acid–water (4:1:5), identification being effected by comparison with the pure compounds. DNP-sarcosine appears behind DNP-threonine.

DNP-β-alanine and γ-DNP-aminobutyric acid form one overlapping spot appearing below that of DNP-glycine. After elution of the whole spot, separation can be effected, by paper electrophoresis at pH 3.9 ((buffer: pyridine–acetic acid–water (30:100:4870)) at 300 V for 4 h in a Durrum type apparatus, pure samples being run at the same time. The increasing order of migration towards the anode is as follows: γ-DNP-amino-butyric acid, DNP-β-alanine, DNP-glycine. By using the system *sec.*-butanol–pH 6 phthalate buffer it is possible to separate DNP-glycine ($R_F = 0.29$), DNP-alanine ($R_F = 0.43$), and DNP-β-alanine ($R_F = 0.52$) (time of development: 40 h) (personal results). The R_F values of γ-DNP-aminobutyric acid in various solvent systems are as follows (KOJIMA *et al.*[40]): *n*-butanol saturated with ammonia: 0.40; *n*-butanol–water–ethanol (4:2:1): 0.90; *n*-butanol–acetic acid–water (4:1:5): 0.95; 0.1 *M* phosphate buffer: 0.66.

β-DNP-amino-isobutyric acid (T-spot) coincides with the spot of DNP-alanine. After elution of this zone, these two compounds can be identified, by one dimensional chromatography in butanol–acetic acid–water (4:1:5), running pure samples side by side on the same sheet. Alanine appears behind β-DNP-amino-isobutyric acid.

DNP-pipecolic acid does not separate from DNP-valine in two-dimensional chromatography. After elution of this zone, these two compounds can be identified with the aid of reference substances by paper electrophoresis with a pH 2.4 *N* acetic acid buffer at 300 V for 5 h in a Durrum-type apparatus. DNP-valine migrates more rapidly than DNP-pipecolic acid.

All these identifications are lengthy and difficult. It is essential that they should be carried out in the presence of the corresponding pure DNP-amino acids used as internal and lateral reference substances.

8. CHROMATOGRAPHY OF WATER-SOLUBLE DNP-AMINO ACIDS

The separation of water-soluble DNP-amino acids depends directly upon the particular applications of the DNP-amino-acid method.

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(a) Water-soluble DNP-amino acids from a total hydrolysate of protein

1. *Dinitrophenylation in aqueous alcoholic medium* (see Section 3, b, 2, p. 68)

The ether-insoluble DNP-amino acids of the medium are α-mono-DNP-arginine and di-DNP-histidine. Di-DNP-histidine can be extracted with ethyl acetate. One may therefore be prompted either to separate α-mono-DNP-arginine and di-DNP-histidine or to chromatograph α-mono-DNP-arginine alone. This problem can easily be solved by one-dimensional chromatography in the "toluene" system (see Fig. 11). In spite

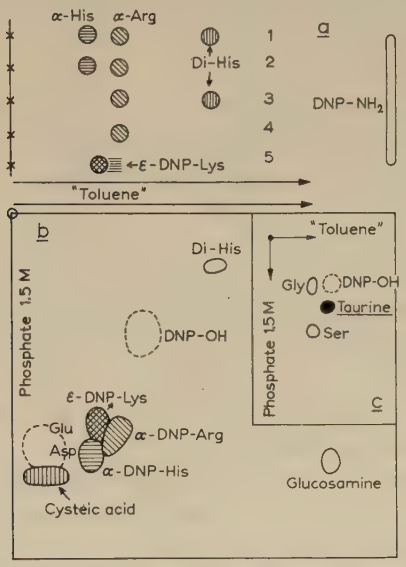

Fig. 11. Chromatography on paper of water-soluble DNP-amino acids. (*a*) One-dimensional chromatography of water-soluble DNP-amino acids ("toluene" solvent system). 1 = mixture of reference substances; 2 = total hydrolysate after dinitrophenylation in aqueous medium (LEVY's technique, Section 3, b, 1, p. 66); 3 = total hydrolysate after dinitrophenylation in aqueous alcoholic medium (Section 3, b, 2, p. 68); 4 = total hydrolysate after extraction of di-DNP-histidine with ethyl acetate (see Table 2); 5 = ε-DNP-lysine (and perhaps other water-soluble dinitrophenyl derivatives) after total hydrolysis of a DNP-protein. (*b*) Two-dimensional chromatogram of water-soluble dinitrophenyl derivatives. Solvent systems: "toluene" and 1.5 *M* phosphate. (*c*) Position of DNP-taurine (water-soluble) on the chromatogram obtained with "toluene" and 1.5 *M* phosphate.

of the apparent simplicity of the problem, numerous technical difficulties may arise in the course of its solution. The variability of the R_F values of these compounds and the presence of coloured artefacts are the main sources of error. It is therefore essential to carry out the one-dimensional chromatography on a separate sheet of Whatman No. 1 paper, and not, as indicated by LEVY[43], on the chromatogram used for the separation of the ether-soluble DNP-amino acids (the spots of the ether- and water-soluble substances being placed at the two ends of the sheet, the strip containing the water-soluble DNP-amino acids is then cut out before developing in the second dimension with 1.5 *M* phosphate).

On this separate sheet it is also essential to place lateral and internal controls

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of α-mono-DNP-arginine and di-DNP-histidine and to apply the Sakaguchi reaction* which gives an orange-red coloration with α-mono-DNP-arginine.

2. *Dinitrophenylation in aqueous medium* (LEVY's technique; see Section 3, b, 1, p. 66)

Under these conditions the water-soluble DNP-amino acids can be α-mono-DNP-arginine and α-mono-DNP-histidine, which cannot be extracted with ethyl acetate. Separation can also be effected by one-dimensional development in the "toluene" system on a separate sheet, in the presence of lateral and internal controls. Finally, it is essential to verify the identity of the dinitrophenyl derivatives, using Sakaguchi's reaction for α-mono-DNP-arginine and Pauly's reaction** for α-mono-DNP-histidine.

(*b*) *Water-soluble DNP-amino acids from a hydrolysate of DNP-protein or of DNP-peptide*

(Problem of identifying an amino-acid residue in terminal position)

1. *Protein*

In the case of a protein, histidine and arginine in N-terminal position can give water-soluble derivatives, di-DNP-histidine (negative Pauly reaction) and perhaps α-mono-DNP-histidine (positive Pauly reaction), and α-mono-DNP-arginine (positive Sakaguchi reaction). In the case of an oxidized protein, cystine if in terminal position is oxidized to cysteic acid, and the water-soluble phase of the hydrolysate of the oxidized DNP-protein may also contain DNP-cysteic acid.

But the hydrolysate necessarily contains other dinitrophenyl derivatives: ε-DNP-lysine (a yellow compound, giving a brown reaction with ninhydrin), imidazole-DNP-histidine (a compound with little colour, giving a brown reaction with ninhydrin and exhibiting a dark fluorescence in Wood's light; negative Pauly reaction), and O-DNP-tyrosine (colourless compound, giving a violet reaction with ninhydrin and a dark fluorescence in Wood's light). Occasionally S-DNP-cysteine may be present.

The problem to be solved may thus be relatively complicated, the more so because the quantities of ε-mono-DNP-lysine are always large in comparison with the quantities of dinitrophenyl derivatives that may be derived from a residue in N-terminal position.

There are several ways in which the problem can be tackled. An attempt may be made to separate all the compounds by a chromatographic technique or the mixture may be simplified before it is subjected to chromatography.

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* *Recommended technique for Sakaguchi's reaction*: spray the chromatogram with a 0.1% solution of 8-hydroxyquinoline in acetone; after drying, spray a solution of sodium hypobromite prepared immediately before use by dissolving 0.2 ml of bromine in 100 ml of 0.5 *N* sodium hydroxide; the spots of DNP-arginine turn pink-red.

** *Recommended technique for Pauly's reaction:* spray the chromatogram with a mixture of equal parts of the following two reagents: *reagent a:* *p*-anisidine 1 g, pure hydrochloric acid 1 ml, absolute ethanol 100 ml; *reagent b:* amyl nitrite 10 g, absolute ethanol to 100 ml; wait 3–5 minutes for the paper to dry at room temperature, then spray with a solution of 1% KOH in ethanol; α-mono-DNP-histidine and histidine give brick-red spots on an orange-yellow background.

References p. 103/104.

(*i*) *Chromatographic separations of water-soluble dinitrophenyl derivatives.* The solvent systems recommended may be either those used in the classical chromatography of the amino acids, or those used in the chromatography of ether-soluble dinitrophenyl derivatives.

Acid butanol systems may profitably be employed: the system butanol–acetic

TABLE 6

R_F VALUES OF WATER-SOLUBLE DINITROPHENYL DERIVATIVES

Whatman No. 4 paper; solvent butanol–acetic acid–water (4:1:5) prepared 24 h before use.

	R_F	*Coulour of spots*	*Reaction with ninhydrin*	*U.V.*
O-DNP-tyrosine	0.84	colourless	violet	dark
α-DNP-arginine	0.81	yellow	no reaction	
ε-DNP-lysine	0.77	yellow	brown	
Leucine*	0.67	—	—	
Imidazole-DNP-histidine	0.57	(?)	brown	dark
Valine*	0.49	—	—	
DNP-cysteic acid	0.42	yellow	—	

* The R_F values of these amino acids are given for purposes of comparison.

TABLE 7

R_F VALUES OF WATER-SOLUBLE DINITROPHENYL DERIVATIVES IN VARIOUS SOLVENT SYSTEMS

	Butanol–formic acid		*Butanol–acetic acid*		*Phenol (NH_3)*
	MARGOLIASH[54]	ACHER[1]	SANGER AND TUPPY[80]	*Personal results*	
Histidine	0.02			0.09	
α-DNP-histidine	0.09			0.57	
Imidazole-DNP-histidine	0.16	0.24	0.57	0.33	
Di-DNP-histidine	0.63	0.75		0.83	0.87
ε-DNP-lysine		0.56	0.77	0.60	0.90
α-DNP-arginine		0.65	0.81	0.65	0.93
DNP-cysteic acid*			0.42	0.45	0.24
Taurine				0.43	

* DNP-cysteic acid is not adsorbed on columns of talc like the other water-soluble dinitrophenyl derivatives (see Section 4, d, 1, p. 73).

acid–water (4:1:5) of PARTRIDGE, prepared 24 h before use (SANGER AND TUPPY[80]) (see Tables 6 and 7 and Fig. 12); the system *n*-butanol–acetic acid–water (3:1:1) (INGRAM AND SALTON[36]) for ascending chromatography*; and the system *n*-butanol–formic acid–water (75:15:10) (MARGOLIASH[54]; ACHER[1]) (see Table 7). In the system *n*-butanol–acetic acid–water (250:60:250 v/v) of WOIWOD[93], δ-mono-DNP-ornithine

* With this system it is possible to separate ε-mono-DNP-lysine and mono-DNP-diaminopimelic acid. These two derivatives can also be separated by electrophoresis on Whatman No. 3 MM paper, in pH 6.4 buffer (pyridine–acetic acid–water, 10:0.4:90) with 20 V/cm for 1 h (INGRAM AND SALTON[36]).

migrates less quickly than ε-mono-DNP-lysine (NEWTON AND ABRAHAM[62]). The variability of the R_F values according to the experimental conditions makes it necessary to use internal and lateral controls and specific colour tests (Pauly and Sakaguchi reactions).

Phenolic solvents can also be used, notably water-saturated phenol in an atmosphere of 3 % ammonia (v/v) and hydrocyanic acid (see Table 7), which permits a satisfactory separation of DNP-cysteic acid from all the other water-soluble dinitrophenyl derivatives. In two-dimensional chromatography (butanol–acetic acid–water

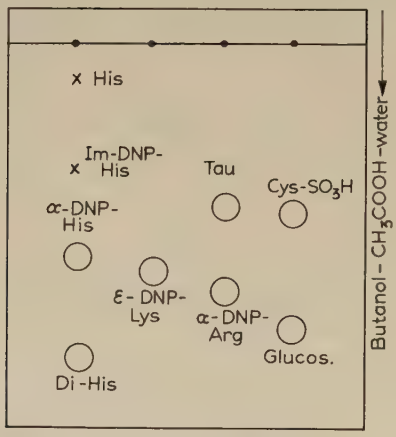

Fig. 12. Paper chromatography of water-soluble DNP-amino acids. Solvent system: butanol–acetic acid–water (4:1:5). The crosses indicate the position of free histidine (His) and imidazole-DNP-histidine (Im-DNP-His), a very faintly yellow-coloured compound. Cys-SO_3H = DNP-cysteic acid.

(4:1:5) in the first dimension and phenol (3 % NH_3) or *m*-cresol–phenol–pH 9.3 buffer (25:25:7)* in the second dimension) DNP-cysteic acid is clearly separated.

The solvent systems described for the ether-soluble dinitrophenyl derivatives can also be employed, especially the system *tert.*-amyl alcohol–pH 6 phthalate buffer of BLACKBURN AND LOWTHER (see Fig. 13) or the "toluene" system of BISERTE AND OSTEUX (see Fig. 11).

Two-dimensional chromatography with "toluene"–1.5 *M* phosphate has also been recommended, but the result is not very satisfactory; the separation of arginine and of ε-mono-DNP-lysine is incomplete** and this solvent pair does not give better results than one-dimensional chromatography.

Paper electrophoresis may also readily solve certain problems. For example, the

* Borate buffer: 200 ml 0.1 *N* boric acid and 113.5 ml 0.1 *N* NaOH. The buffer is sprayed uniformly on the paper before developing with the second solvent, except the zone of the chromatogram where the amino acids separated by the first solvent are to be found (LEVY AND CHUNG[44]). In the chromatographic map of amino acids and water-soluble DNP-amino acids given by FRAENKEL-CONRAT, HARRIS AND LEVY[28], the position of di-DNP-histidine is incorrect (see Fig. 12).

** The chromatographic map published by DAVIES AND HARRIS[22] is incomplete and to a certain extent incorrect.

separation of α-DNP-arginine and of ε-DNP-lysine can be effected by electrophoresis on paper in *N* ammonia in a Durrum-type apparatus (see Fig. 14). In the same way DNP-cysteic acid and ε-mono-DNP-lysine, extracted from the hydrolysate with *n*-butanol, can be separated by electrophoresis on Whatman No. 3 paper moistened with a 0.1 *M* phosphate buffer of pH 7.0 (ANFINSEN, SELA AND TRITCH[3]).

(*ii*) *Simplification of the mixture of water-soluble dinitrophenyl derivatives before chromatography.*

(α) *α-DNP-arginine.* The amino acids can be eliminated from the hydrolysate (all the constituent amino acids, plus some molecules of lysine or of histidine that

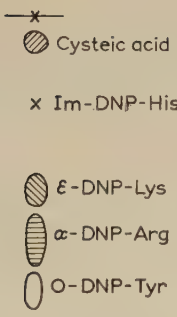

Fig. 13. Chromatogram of the water-soluble DNP-amino acids. Solvent system *tert.*-amyl alcohol–phthalate of BLACKBURN AND LOWTHER (according to FRAENKEL-CONRAT, HARRIS AND LEVY[28]).

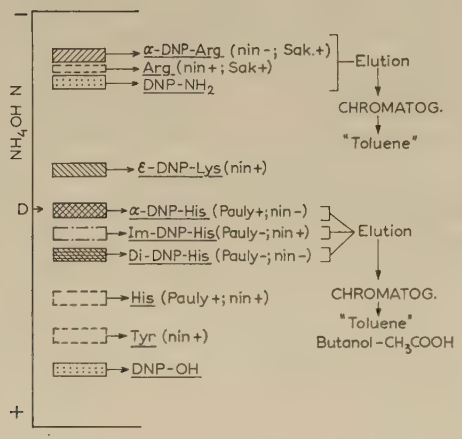

Fig. 14. Electrophoretic separation of water-soluble DNP-amino acids. Paper Whatman No. 1 or No. 3; buffer: *N* NH_4OH; electrophoresis apparatus: Durrum type. D = starting point of the electrophoresis; nin = ninhydrin reaction; Sak = Sakaguchi reaction; Pauly = Pauly reaction; DNP-OH = dinitrophenol; DNP-NH_2 = dinitraniline. The rectangles enclosed by dotted lines are not yellow upon direct examination. After elution of the various spots separated by electrophoresis, the eluates are investigated by one-dimensional chromatography.

are not reactive and have not been dinitrophenylated) on a column of talc (SANGER, see Section 4, d, 1, p. 73). The eluate from the talc column which contains the water-soluble dinitrophenyl derivatives, is treated with fluorodinitrobenzene (ROVERY, FABRE AND DESNUELLE[75]). Under these conditions, ε-mono-DNP-lysine is converted to di-DNP-lysine, O-DNP-tyrosine to di-DNP-tyrosine and imidazole-DNP-histidine to di-DNP-histidine, while the dinitrophenyl derivative of arginine (α-mono-DNP-arginine) remains unchanged. After this second dinitrophenylation, ether extraction of the residue removes di-DNP-lysine and di-DNP-tyrosine, while the aqueous phase contains di-DNP-histidine and α-mono-DNP-arginine derived from a terminal amino acid of the peptide chain. The aqueous phase is chromatographed using the system butanol–

acetic acid–water (4:1:5) (see above) and certain identification of α-mono-DNP-arginine is achieved by means of the Sakaguchi reaction.

Another procedure has also been proposed by BAILEY[4]. Here ε-mono-DNP-lysine is converted into ε-mono-DNP-α-methoxycarbonyllysine (ε-DNP-α-MC-lysine), which is ether-soluble. The method is as follows. After extraction of the ether-soluble DNP-amino acids, the aqueous phase (equivalent to 0.2 g of protein) is evaporated to dryness, dissolved in 0.1 *N* hydrochloric acid and passed over a column of talc (see BAILEY AND BETTELHEIM[5] and Section 4, d, 1, p. 73). The water-soluble amino acids are eluted with ethanol–hydrochloric acid (ethanol: 4 vol.; *N* HCl: 1 vol.). They are dissolved in 2–3 ml of a bicarbonate–carbonate mixture of pH 8.9 (mixture of 20 ml of 10 % sodium bicarbonate and 5 ml of 10 % sodium carbonate). The solution is brought to 20°, and four portions, each of 0.02 ml, of methoxycarbonyl chloride are added at intervals of 10 min with vigorous agitation. The mixture is acidified with hydrochloric acid and extracted four times with ether to eliminate ε-DNP-α-MC-lysine. The aqueous phase is chromatographed as above.

(β) *Derivatives of histidine.* To solve this problem, it is impossible to use the technique described for α-DNP-arginine, because in the course of the second dinitrophenylation all the derivatives of histidine in intrapeptide or terminal poisition may be converted to di-DNP-histidine.

In order to tackle this difficult problem one can resort to selective extractions, such as continuous ether extraction of the aqueous residue according to the methods described and with the apparatus recommended by MILLS[58], or extraction with ethyl acetate.

The one-dimensional chromatography in the presence of internal and lateral controls, with the application of specific colour tests (Pauly reaction, ninhydrin reaction) constitutes the final stage of identification, which is not invariably crowned with success.

Conclusion: the identification of water-soluble dinitrophenyl compounds derived from amino acids in N-terminal position is always difficult, and before deciding that they are present it is essential to secure sufficient and indisputable supporting evidence. It is necessary to employ a number of solvent systems and paper electrophoresis (Fig. 14).

(*iii*) *Artefacts in the water-soluble fraction.* A certain number of artefacts can be found in the water-soluble fraction.

BAILEY[4], in particular, has drawn attention to the presence of an impurity with a brown colour, which can easily be separated from α-mono-DNP-arginine (if the latter is present) by passing it over a small column of acid silica with methyl ethyl ketone (SANGER[76]).

We have also fairly frequently found yellow-coloured spots of an unknown nature on the chromatograms. Therefore it is absolutely imperative to use lateral and internal controls and specific colour tests in order to localize the water-soluble DNP-amino acids.

We also call attention to an artefact that can interfere with the identification of

DNP-cysteic acid*. In two-dimensional chromatography with butanol–acetic acid and aqueous phenol, the R_F values of these two compounds, according to THOMPSON, are as follows:

	R_F artefact	R_F DNP-cysteic acid
Butanol–acetic acid	0.29	0.18
Phenol	0.58	0.38

2. *Particular problems of separation*

(*i*) *Separation of monodinitrophenyl derivatives of diamino acids.* In the chemistry of bacterial polypeptides not only lysine can be found, but also ornithine and α,γ-diaminobutyric acid. Separation of the dinitrophenyl derivatives of these diamino acids is very difficult: we have mentioned above the impossibility of separating the

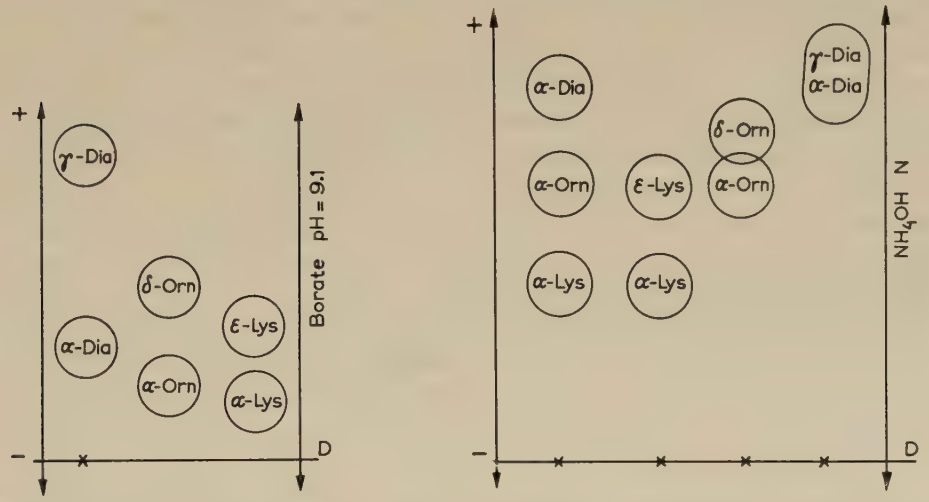

Fig. 15. (*a*) Paper electrophoresis of dinitrophenyl derivatives of diamino acids. Electrophoresis in a Durrum-type apparatus; buffer: 0.02 *M* borate of pH 9.1; paper: Arches 304 or Whatman No. 3; 10 V/cm; 17 h. Distance travelled by α-DNP-diaminobutyric acid = 6 cm; by γ-DNP-diaminobutyric acid = 16 cm. (*b*) Paper electrophoresis of dinitrophenyl derivatives of diamino acids. Electrophoresis in a Durrum-type apparatus; paper: Whatman No. 3; buffer: *N* NH_4OH; 8–9 V/cm; 16 h. Distance travelled by α-DNP-lysine = 5 cm.

ether-soluble dinitrophenyl derivatives of lysine, ornithine and α,γ-diaminobutyric acid. The separation of monodinitrophenyl derivatives, whether substituted in position α or in position ε, is also very difficult. The procedure suggested by NEWTON AND ABRAHAM[62] for the separation of ε-mono-DNP-lysine and δ-mono-DNP-ornithine with butanol–acetic acid–water (250:60:250) is not very efficient, even in the presence of lateral controls.

More satisfactory results can be obtained by paper electrophoresis.

(*a*) *Separation of ε-mono-DNP-lysine, δ-DNP-ornithine and γ-mono-DNP-diaminobutyric acid.* Electrophoresis with 0.02 *M* borate buffer at pH 9.1 (LOCKHART

* This problem has recently arisen in connexion with the terminal amino acids of serum albumin. THOMPSON has shown that the substance identified by TITANI *et al.*[89] was an artefact.

AND ABRAHAM[50]) on Arches 304 or Whatman No. 3 paper, in a Durrum-type apparatus; 10 V/cm for 17 h (see Fig. 15a).

(β) *Separation of α-mono-DNP-lysine, α-mono-DNP-ornithine and α-mono-DNP-diaminobutyric acid.* Electrophoresis with *N* ammonia as electrolyte on Whatman No. 3 paper in a Durrum-type apparatus; 8–9 V/cm for 16 h (see Fig. 15b). Paper electrophoresis does not separate α-mono-DNP-diaminobutyric acid from γ-mono-DNP-diaminobutyric acid, or α-mono-DNP-ornithine from δ-mono-DNP-ornithine. ε-Mono-DNP-lysine can be confused with α-mono-DNP-ornithine.

A combination of two one-dimensional electrophoreses, one with *N* NH_4OH as electrolyte and the other, following elution, in a 0.02 *M* borate buffer at pH 9.1, makes a solution of all these problems possible.

(γ) *Separation of monodinitrophenyl derivatives substituted at position α or ω.* It is not possible to separate these compounds with the chromatographic solvent systems. The separation of derivatives of one and the same series: α-mono-DNP-lysine and ε-mono-DNP-lysine; α-mono-DNP-ornithine and δ-mono-DNP-ornithine; α-mono-DNP-diaminobutyric acid and γ-mono-DNP-diaminobutyric acid, is easily achieved by paper electrophoresis in 0.02 *M* borate buffer (pH 9.1) (see Fig. 15a).

(*ii*) *Separation of DNP-glucosamine and DNP-chondrosamine.* These two derivatives can be readily separated in the solvent system butanol–ethanol–water (4:1:5) on Whatman No. 1 paper. The R_F values of these substances are: DNP-glucosamine 0.75; DNP-chondrosamine 0.61 (KENT *et al.*[38]).

On the two-dimensional chromatogram with "toluene"–1.5 *M* phosphate, DNP-glucosamine is very clearly separated from all the other derivatives (see Fig. 11).

(*iii*) *DNP-taurine.* This can also be chromatographed in the system butanol–acetic acid and in the "toluene" system (see Fig. 11).

(*c*) *Separation of the water-soluble DNP-amino acids (S-DNP-Cys, O-DNP-Tyr, ε-DNP-Lys) from other DNP-amino acids and from free amino acids by paper electrophoresis* (Fig. 16)

High voltage electrophoresis (6000 V, 6–8 mA) was carried out for 180 min with cooling in the apparatus of KICKHÖFEN AND WESTPHAL[38a], on sheets 80 cm × 14 cm, using a volatile pH 1.93 buffer (formic acid–acetic acid) (ZUBER, TRAUMANN AND ZAHN[100]).

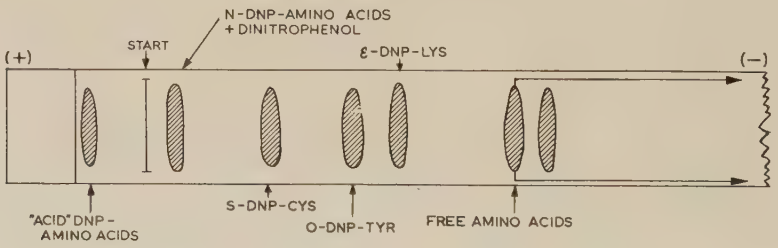

Fig. 16.

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9. CHROMATOGRAPHY OF DNP-PEPTIDES

The chromatography of DNP-peptides poses very special problems and it is difficult to present a precise scheme of separation valid for all cases*.

The DNP-peptides are mostly derived from an acid or enzymic partial hydrolysate of a DNP-protein. The partial hydrolysate can be extracted successively with ether (3 times), with ethyl acetate (3 times) and with *n*-butanol (once). The residual aqueous phase may still contain dinitrophenyl peptides (LI *et al.*[49]).

The behaviour of DNP-oligopeptides resembles to a certain extent that of the DNP-amino acids. On the other hand separation of the DNP-peptides is rather restricted because of their insolubility in organic solvents or in acid or alkaline aqueous media. Thus all the solvent systems described for the amino acids can be tried.

The following solvent system has also been employed, on Whatman No. 3 paper: *sec.*-butanol–*tert.*-butanol–2,4,6-collidine–ammonia–glacial acetic acid–water (60:20:20:2:0.25:100 v/v) (REDFIELD AND ANFINSEN[70]).

Interesting combinations have been proposed by COLE, LI, HARRIS AND PON[19] and by LI, COLE, CHUNG AND LEONIS[49].

1. *Ether-soluble DNP-peptides*

One-dimensional separations have been effected in the systems *tert.*-amyl alcohol–isoamyl alcohol–3 % aqueous NH_4OH (1:1:2); *tert.*-amyl alcohol–3 % aqueous NH_4OH (1:1); *tert.*-amyl alcohol–isoamyl alcohol–3 % aqueous NH_4OH (2:1:3).

2. *DNP-peptides soluble in ethyl acetate*

Two-dimensional chromatography may be carried out with *tert.*-amyl alcohol–3 % aqueous NH_4OH (1:1) in the first dimension and a 0.8 *M* phosphate of pH 6.7 in the second dimension; or one-dimensional chromatography in the system *tert.*-amyl alcohol–isoamyl alcohol–3 % ammonia (2:1:3).

3. *DNP-peptides soluble in n-butanol and in the aqueous phase*

One-dimensional development in the system *tert.*-amyl alcohol–isoamyl alcohol–3 % ammonia (2:1:3) and 1.6 *M* phosphate of pH 7 has been employed.

The possibilities of separation offered by paper electrophoresis should also be pointed out, especially with a buffer saturated with urea, which reduces the adsorption of DNP-peptides on the paper (8 *M* solution of urea and 0.04 *M* collidine–acetate buffer of pH 7.9) (REDFIELD AND ANFINSEN[70]). High potential electrophoresis was carried out with 800 V on Whatman No. 3 paper under toluene (MICHL[56]).

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* THOMPSON[86] and MCFADDEN AND SMITH[53] have proposed schemes for fractionation on columns of buffered Celite. Counter-current distribution can also give very satisfactory results (BATTERSBY AND CRAIG[6]; REDFIELD AND ANFINSEN[70]).

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10. QUANTITATIVE CHROMATOGRAPHY OF DNP-AMINO ACIDS

Chromatography of DNP-amino acids can easily be rendered quantitative. After elution of the spot, the eluate is determined by direct spectrophotometry.

(*a*) *Determination of amino acids from a hydrolysate*

(LEVY'S method[43])

1. *Elution of the spots*

For the hydrolysis and the formation of the DNP-amino acids, see Sections 3, a and 3, b, pp. 66 *et seq.* After chromatography of the ether-soluble DNP-amino acids (two-dimensional chromatogram of the DNP-amino acids corresponding to 0.1–0.2 mg of protein) and of the water-soluble amino acids (one-dimensional chromatogram of the DNP-amino acids corresponding to 0.2–0.4 mg of protein) (see pp. 77, 89), the spots are carefully cut out and the corresponding pieces of paper placed in small centrifugation tubes with 4 ml of 1 % sodium bicarbonate. The tubes are heated to 50–55° in a water bath for 20 min to complete the elution, then shaken out and centrifuged. Three pieces of paper serving as blanks (3 × 4 cm) are also cut out of the chromatogram, at places close to DNP-leucine and the DNP-dicarboxylic acids and above dinitrophenol. After cooling, the eluate is poured into a quartz cell 1 cm thick.

2. *Determination*

The optical density is measured in the spectrophotometer at 360 mμ (except for proline and hydroxyproline where the reading is made at 385 mμ, for DNP-peptides at 350 mμ and for ε-mono-DNP-lysine at 390 mμ), against a blank containing water.

The optical densities of the tubes containing the paper blanks are also measured: the results are of the order of 0.001–0.002 per cm^2. Appropriate corrections are made for each spot according to its area. The total optical density of the aspartic acid + glutamic acid spot is measured.

After a second development with the combination "toluene–2.5 *M* phosphate", the proportions of glutamic acid and aspartic acid can be determined.

For the determination of O-DNP-tyrosine and of ε-DNP-lysine see FRITZE AND ZAHN[29b].

For the determination of S-DNP-cysteine: At room temperature and at a pH lower than 5.5 fluorodinitrobenzene reacts only with the SH group of cysteine. After hydrolysis of the protein, S-DNP-cysteine can be separated by paper electrophoresis (see Fig. 16) and determined spectrophotometrically at 330 mμ (see ZUBER, TRAUMANN AND ZAHN[100]).

3. *Expression of the results*

Recoveries in absolute quantities vary from one chromatogram to another. The results are therefore expressed on a common basis in molar fractions.

In order to convert the optical densities into molar ratios they are multiplied by

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the factors of LEVY (see Table 8)*, which, within the accuracy of the method (± 4 %), are independent of the composition of the mixture analysed.

TABLE 8

CORRECTION FACTORS USED IN THE DETERMINATION OF DNP-AMINO ACIDS

	LEVY'S *factors*[43]	BROMER'S *factors*[17]
Glycine	1.03	1.33
Serine	0.97	1.32
Threonine	1.02	1.22
Proline	0.93	—
Alanine	1.09	1.28
Methionine	1.21	2.12
Valine	0.99	1.20
Leucine	1.10	—
Phenylalanine	1.03	1.20
Lysine	0.64	0.70
Arginine	1.06	1.38
Histidine	1.62	2.19
Aspartic acid	0.99	1.10
Glutamic acid	0.94	1.20
Cystine	0.56	—
Tyrosine	1.54	1.60

The chromatograms are run in triplicate. Several hydrolysates obtained after different times of hydrolysis can be analysed (hydrolysis for 24 and 48 h, for example).

All the optical density readings, multiplied by LEVY's factors, are added up. For each sum thus obtained, a factor can be calculated for which this sum is equal to one.

Eliminating deviating values, which undoubtedly arise from technical errors or chromatographic imperfections, one can calculate an average for all the results and express them in molar ratios (see Table 9). In general, the most variable results are those for tyrosine (variability in the synthesis), methionine (possible degradation) and histidine (variability in the synthesis and difficulty of extraction). According to LEVY, the molar ratios are reproducible to within about 2–3 %.

On the basis of these values the number of amino acid residues per minimum molecular weight of the protein can be calculated by dividing the average values of the molar ratios by that one among them which leads to the result nearest to a whole number for the greatest possible number of amino acids (see Table 9, where the values are divided by that of leucine.) The values for serine are corrected (10 %) because of the partial decomposition of this amino acid after 24 h of hydrolysis (REES[71]). These values are then rounded off to the nearest whole number (see Table 9).

It is evident that, from the results expressed as molar ratios, the number of

* LEVY determined these factors for hydrolysates of insulin. They are not necessarily entirely valid for other proteins. BROMER *et al.*[17], for instance, have calculated new factors for glucagon. As a first approximation they apply the factors of LEVY to the protein hydrolysate. On the basis of the quantitative data thus obtained, they prepare a mixture of amino acids resembling glucagon, submit it to the operations of hydrolysis and treat it as a protein hydrolysate. In this way new factors of destruction can be calculated. This operation is repeated several times to obtain a greater precision in the correction factors. On the whole BROMER's factors are higher (about 17%) than those of LEVY. In particular, histidine and methionine show greater losses than those indicated by LEVY.

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TABLE 9

AMINO-ACID COMPOSITION OF A PREPARATION OF α-CORTICOTROPIN

(According to LEVY *et al.*[46])

Amino acid	*Results as molar frations*			*Mean molar reaction*	*Deviation from mean (%)*	*Minimal residues per molecule*	*Residues to nearest integer*
	I	*II*	*III*				
Aspartic acid	0.0392	0.0503	0.0491	0.0462	10.1	1.72	2
Glutamic acid	0.1345	0.1273	0.1282	0.1300	2.3	4.85	5
Serine	0.0689	0.0690	0.0702	0.0694	0.9	2.85	3
Glycine	0.0837	0.0818	0.0842	0.0832	1.2	3.10	3
Alanine	0.0747	0.0753	0.0753	0.0751	0.3	2.80	3
Proline	0.1107	0.1084	0.1087	0.1093	0.9	4.08	4
Valine	0.0798	0.0798	0.0816	0.0804	1.0	2.99	3
Methionine	0.0235	0.0211	0.0212	0.0219	4.7	0.82	1
Leucine	0.0291	0.0246	0.0268	0.0268	5.7	1.00	1
Phenylalanine	0.0822	0.0809	0.0823	0.0818	0.8	3.05	3
Tyrosine	0.0608	0.0647	0.0607	0.0621	2.8	2.32	2
Lysine	0.1049	0.1073	0.1018	0.1047	1.8	3.91	4
Histidine	0.0268	0.0272	0.0273	0.0271	2.1	1.01	1
Arginine	0.0810	0.0821	0.0825	0.0819	0.4	3.06	3
Tryptophan*							1
Amide-NH_3							2
Total	0.9998	0.9998	0.9999	0.9999	2.5	37.6	41

* Determined by the method of GOODWIN AND MORTON[30].

residues of amino acids per 100 amino acids of the molecule can also be obtained. To this end the readings of the optical density multiplied by LEVY's factors are added up. For each sum a correction factor can be found such that the sum becomes equal to 100 (instead of 1 in the preceding calculation). By multiplying all the numbers by this factor, one can then express the results on the basis of 100 amino-acid residues.

Taking into account the molecular weight of the molecule, the number of residues of each amino acid present in the entire molecule can be calculated. The number of residues of each amino acid per 100 amino acids, is multiplied by the molecular weight of the corresponding amino acid, and all the results are added together. From the sum thus obtained the value of 1782, corresponding to 99 molecules of water, is deducted. The ratio is calculated between the actual molecular weight and the partial molecular weight thus calculated. The number of residues per 100 amino acids multiplied by this ratio gives the number of residues for the whole molecule. It then suffices to round off the values to know the number of amino-acid residues per molecule (see Table 10). An identical calculation can also be made by taking into account the number of amino acids found for the minimum molecular weight.

Finally, if it is necessary to estimate the absolute quantities of the amino acids present in the mixture, two methods can be applied (LEVY[43]): the less accurate one (2–5 %) consists in a direct conversion of the optical density read into micromoles by means of a set of millimolar extinction coefficients. These have the values $15.6/F$

where F represents the factors given in Table 8. The lack of accuracy of this procedure arises mainly from differences in the recovery of coloration from several chromatograms. These differences are of no importance when determining the molar ratios, for these variations affect all the amino acids in a comparable manner.

TABLE 10

COMPOSITION OF HAEMERYTHRIN OF *Sipunculus nudus*

(Personal results)

	Number of residues per 100 amino acids	*Number of residues calculated from the values of column 1 on the basis of a mol. wt. of 66,000*	*Number of residues in haemerythrin (values in whole numbers)*
Gly	4.42	25.37	25
Ser	4.22	24.33	24
Thr	4.33	24.85	25
Pro	5.62	32.25	32
Ala	7.40	42.47	42
Met	0.38	2.18	2
Val	6.80	39.03	39
Leu	14.10	80.93	81
Phe	11.62	66.69	67
Lys	8.15	46.78	47
Tyr	3.80	21.80	22
Arg	2.42	13.89	14
His	4.36	25.02	25
Asp	13.82	79.32	79
Glu	8.10	46.49	46
Cys	0.34	1.94	2
			572 residues

TABLE 11

APPROXIMATE DESTRUCTION OF DINITROPHENYL DERIVATIVES DURING HYDROLYSIS

(According to PORTER[66])

	Time (h)	*Quantity remaining (%)*
DNP-alanine	12	80
DNP-arginine	12	90
DNP-aspartic acid	24	60
Di-DNP-cystine	12	25
DNP-glutamic acid	12	75
DNP-glycine	8	40
DNP-hydroxyproline	4	40
DNP-leucine	12	80
DNP-isoleucine	12	80
Di-DNP-lysine	8	95
ε-DNP-lysine	12	95
DNP-methionine	12	75
DNP-phenylalanine	12	70
DNP-proline	2	10
DNP-serine	12	90
DNP-threonine	24	90
DNP-tryptophan	12	90
DNP-tyrosine	12	75
DNP-valine	12	80

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The second method consists in combining the more accurate determination (2–3 %) of the molar ratios with a determination of the total quantity of amino acids present in the mixture. This can be deduced from the consumption of sodium hydroxide during the course of the coupling reaction (see LEVY[43] and LEVY *et al.*[46]).

(*b*) *Determination of a DNP-amino acid in N-terminal position*

A certain amount of terminal DNP-amino acid is destroyed during hydrolysis (see Table 11); and, furthermore, the chromatographic recoveries also involve losses (for example, chromatographic recovery of DNP-aspartic acid and of DNP-glutamic acid: 90 %; of di-DNP-lysine: 80 %; of DNP-cysteic acid: 90 %) (REDFIELD AND ANFINSEN[70]).

It is therefore necessary to take these losses into account when making the determinations. In order to estimate the losses, it is recommended to submit a known

TABLE 12

RECOVERY (%) OD DNP-SERINE AFTER HYDROLYSIS IN THE PRESENCE OF α-CORTICOTROPIN AND OF ITS DINITROPHENYL DERIVATIVES

(According to LEVY AND LI[47])

Experiment No.	*Quantity of product submited to hydrolysis*			*Recovery of DNP-serine* (%)*
	DNP-serine (μmoles)	*α-Cortico-tropin (μmoles)*	*DNP-α-cortico-tropin (μmoles)*	
1	1.0	0	0	86
1	1.0	0	1.0	93
2	0.2	0	0	69–67
2	0.2	0.2	0	52–47
2	0.2	0	0.2	70–78

* Recovery of non-hydrolysed DNP-serine = 93 %.
Expt. 1: hydrolysis for 7 h at 105° with 5.7 *N* HCl (in a sealed tube under vacuum).
Expt. 2: hydrolysis for 16 h at 110° with 5.7 *N* HCl.

quantity of the corresponding DNP-amino acid to hydrolysis under the same conditions, and at the same time as the protein or the DNP-protein studied. These destruction coefficients should be determined several times. As an example, the percentages of recovery of DNP-serine hydrolysed in the presence of α-corticotropin and of its dinitrophenyl derivative are given in Table 12. It is remarkable to note that the destruction of DNP-serine, which is increased in the presence of corticotropin, is diminished in the presence of DNP-corticotrophin (see also the experiments of DESNUELLE *et al.*[24] on DNP-aspartic acid from serum albumin).

Interpretation of the results of the determination of an amino acid in terminal position is very often difficult: in spite of the aforesaid corrections, the quantity recovered may represent only a fairly small part of the initial molecule.

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THE CHROMATOGRAPHY OF THE FLAVONOID PIGMENTS

J. B. HARBORNE
John Innes Horticultural Institution, Hertford (Great Britain)

1. INTRODUCTION

Paper partition chromatography was first applied to the separation of flavonoid pigments by BATE-SMITH[1,2] in 1948. Since then, it has been used increasingly in the study of these compounds, which are ideally suited to this particular technique. The flavonoids have just the right range of solubility characteristics for ease of separation and most of them possess characteristic colours on paper in visible or ultraviolet light. Paper chromatography has thus provided the first satisfactory means of surveying plant material for the presence of these water-soluble pigments[3,4]. It has been particularly valuable for separating the components of mixtures of flavonoids, which occur in some plants (*e.g.*[5,6]). Large scale separations have been carried out by means of adsorption chromatography on cellulose or magnesol columns[7,8]. But it is in the characterisation and elucidation of structure of unknown flavonoids on a micro-scale that chromatography has been so successful[5,6,9,10]. The identification of flavonoids by means of chromatography is therefore the major topic of this review. A large number of R_F values are given and some new ones determined in this laboratory are included.

The term flavonoid is used here to describe that group of water-soluble phenolic pigments sometimes known as the anthoxanthins. These are the widely occurring flavones and flavonols and the less frequently distributed flavanones, isoflavones, chalcones and aurones. Flavans (monomeric leucoanthocyanins and catechins) and other related substances may also be considered to fall into this category. The chromatography of the anthocyanins, which are associated with and are structurally related to the anthoxanthins, has already been discussed[11].

In order to be able to relate R_F value to structure (section 4), a brief consideration of the chemical structure of the flavonoids must be made. The formulae of some of the commonly occurring compounds are shown in Fig. 1. Examples are included of all the main classes of flavonoids, which generally differ from each other in the oxidation level of the central pyran ring. Within these classes, structural variation is mainly confined to differences in the number and position of hydroxyl, methyl and glycosyl substituents. There are also a considerable number of rare flavonoids, with additional substituents (*e.g.* methylene-dioxy groups, isoprene side chains, etc.). Their structures are shown in a recent review of the naturally occurring flavonoid pigments by GEISSMAN AND HINREINER[12].

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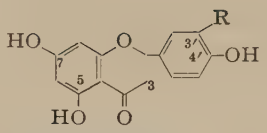
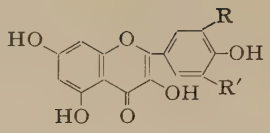
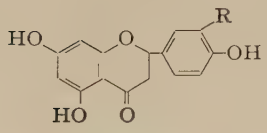


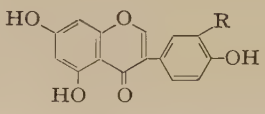
Flavones: apigenin (R = H), luteolin (R = OH)



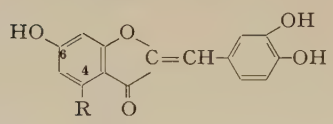
Flavonols: kampferol (R = R′ = H), quercetin (R = OH, R′ = H), myricetin (R = R′ = OH)



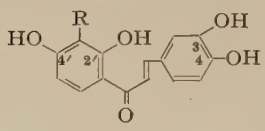
Flavanones: naringenin (R = H), eriodictyol (R = OH)



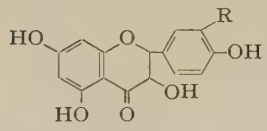
Isoflavones: genistein (R = H), orobol (R = OH)



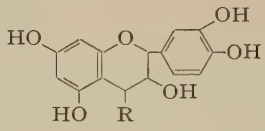
Aurones: sulphuretin (R = H), aureusidin (R = OH)



Chalcones: butein (R = H), okanin (R = OH)



Flavanonols: dihydrokampferol (R = H), dihydroquercetin (R = OH)



Flavans: catechin (R = H), leucocyanidin (R = OH)

Fig. 1. Commonly occurring flavonoids.

2. CHROMATOGRAPHIC TECHNIQUES

BATE-SMITH[2,13] measured the R_F values of thirty-six flavones in two solvent systems. WENDER and his associates[14,15] chromatographed a larger selection of compounds in eleven solvent systems and detected the flavone spots on paper with seven chromogenic sprays. Numerous other workers (*e.g.* PARIS[16], GEISSMAN[17]) have described procedures for the chromatography of the flavones. Brief summaries have appeared in manuals of chromatography[18,19].

Extraction

Flavones are commonly extracted from fresh plant material with hot 70 % ethanol, 95 % ethanol, methanol or water. By plunging the plant material into boiling solvent, the enzymic hydrolysis of glycosides can be kept to a minimum[20]. The extracts are filtered, concentrated to a small volume *in vacuo*, re-filtered and applied by means of a capillary or a micropipette to the chromatography paper. Leaf extracts are first washed with petroleum ether to remove chlorophyll because this compound may cause

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flavones to streak on chromatograms. Samples of flavones are dissolved in ethanol or 70 % ethanol, if necessary by heating, before being applied to paper.

Filter paper

Whatman No. 1 paper (chromatography grade) is widely used for the chromatography of flavones. Thicker papers (Whatman No. 3 or 3 MM) are suitable for small scale separations. Papers buffered either with borate or phosphate[21] have been used by NORDSTRÖM AND SWAIN[5] for the separation of partially methylated flavones. SEIKEL[22] has pointed out that the R_F value varies with the pH of the buffer. This pH should always be given.

Solvent systems

A very large number of solvent systems have been used for the separation of flavonoids on paper.

(1) *Aqueous solvents*. Water is itself an excellent solvent for catechins[23] and flavonol glycosides[24]. It is useful for separating many glycosides from their aglycones, which are usually immobile. Furthermore, those aglycones which travel in water (*e.g.* flavanones) can be separated from those that do not (*e.g.* flavones, flavonols). Some compounds tend to streak with distilled water but this can be minimized by adding acetic acid (2 % v/v)[24] or inorganic salt (3 % w/v sodium chloride)[22]. Stronger mixtures of acetic acid with water are most valuable for separating flavone and flavonol aglycones[4]; the Forestal solvent system (acetic acid–conc. HCl–water, 30:3:10, by vol.), 30 % acetic acid and 60 % acetic acid are examples.

(2) *Alcoholic solvents*. The upper phase of the mixture of *n*-butanol–acetic acid–water (4:1:5, by vol.) (designated as BAW) is most valuable for separating all types of flavonoids, both glycosides and aglycones. Single phase mixtures of these solvents, *i.e.* the proportions 6:1:2[5] or *n*-butanol–27 % acetic acid (equal volumes)[6] have also been used. Alcohol–acetic acid mixtures are sensitive to small temperature changes and alter in composition with age. R_F values determined in BAW often vary more than those measured in other kinds of solvents (*e.g.* aqueous systems).

Other solvent systems containing alcohols have been used to a lesser extent. Examples are *n*-butanol–water (1:1, v/v; upper phase)[14], water-saturated *sec.*-butanol[25] and *n*-propanol–acetic acid–water (1:1:1, by vol.)[26].

(3) *Phenolic solvents*. Phenol or *m*-cresol saturated with water and *m*-cresol–acetic acid–water (50:2:48, by vol.)[2] give a greater spread of R_F values and alter the relative positions of flavones on chromatograms, as compared with BAW. A certain amount of streaking takes place on papers developed with phenolic solvents. Owing to their intense absorption in the ultraviolet, phenols are not suitable solvents for use in purifying flavones on paper for spectral measurements.

(4) *Hydrocarbon solvents*. Benzene–ligroin, b.p. 85–105°–methanol–water (50:50:1:50, by vol.) and related systems devised by LINDSTEDT AND MISIORNY[27] are reported to give excellent separation of flavonoids containing one to three hydroxyl groups, *e.g.* those present in the heartwood of pines. On the other hand mixtures of

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chloroform–isopropyl alcohol–water, chloroform saturated with water and ethyl acetate saturated with water cannot be recommended for general use, as considerable streaking occurs in these systems[15].

Methods of development

The usual technique of one-dimensional chromatography by descent is commonly used. Suitable solvent pairs for two-dimensional chromatography are BAW and 2 % acetic acid[10], *n*-butanol–27 % acetic acid (1:1, v/v) and *m*-cresol–acetic acid–water (50:2:48, by vol.)[6,17], and ethyl acetate–acetic acid–water (50:2:50, by vol.) and BAW[28]. Circular paper chromatography is another technique which can be used with flavones[29]. Paper electrophoresis will separate flavones on Whatman No. 4 paper in *n*-butanol–ethanol–borate buffer (1:1:1, by vol.)[30].

Visualisation

Some flavonoids (*i.e.* aurones and chalcones) can be seen on paper chromatograms as yellow spots in daylight. In ultraviolet light the majority appear as intensely coloured spots, which may be fluorescent. Flavonoids containing free phenolic groups undergo a characteristic and reversible colour change when the papers are fumed with ammonia vapour in ultraviolet light. Some compounds (*e.g.* flavans, isoflavones) can only be detected by means of chromogenic sprays. Among the most useful of these are 5 % ethanolic aluminium chloride[31], 5 % aqueous sodium carbonate, 1 % sodium borohydride in isopropanol[32] and a fresh mixture of equal volumes of 1 % aqueous ferric chloride and 1 % aqueous potassium ferricyanide[33]. After spraying with aluminium chloride or carbonate, the chromatograms should be dried and examined in ultraviolet light, as these reagents impart a fluorescence to many flavonoids. The response of a typical member of each class of flavonoid compound to these different methods of detection is shown in Table 1.

Flavonoids in the same structural class often give characteristic colour reactions. For example, only flavanones form red or magenta colours on reduction with sodium borohydride and subsequent exposure to acid vapour[32]. On paper flavonols and flavones give a particularly distinctive range of colours in the ultraviolet, with and without ammonia vapour[4]. Flavones with a free 3-hydroxyl group (*i.e.* flavonol aglycones) are characteristically bright yellow whereas those without one (*i.e.* flavonol 3-glycosides and ordinary flavones) are dull brown in ultraviolet light. Flavonols without a 5-hydroxyl group (*e.g.* fisetin, robinetin) or with a methylated 5-hydroxyl group (*e.g.* azaleatin) have a greenish-yellow or yellow fluorescence. Flavonols with both the 3- and 5-hydroxyl groups substituted (*e.g.* kampferol tetramethyl ether) fluoresce blue[34,35].

In practice, it is not always possible to classify flavonoids on the basis of their colour reactions alone. This is because some are exceptional in their behaviour. Some of the highly hydroxylated flavonols (*e.g.* quercetagetin, 3,5,6,7,3′,4′-hexahydroxyflavone) are dull brown rather than bright yellow in ultraviolet light[4]. Other flavonols, *i.e.* those with hydroxyl groups in the 3′, 4′ and 5′ positions such as myricetin, give a

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TABLE I

THE COLOUR REACTIONS OF FLAVONOIDS ON PAPER

Reagent	*Light source*	*Class of flavonoid*					
		Flavone	*Flavonol*	*Isoflavone*	*Flavanone*	*Aurone*	*Chalcone*
None	visible	pale yellow	pale yellow	colourless	colourless	bright yellow	yellow
None	ultraviolet	dull brown	bright yellow	faint purple	colourless	bright yellow	dull brown
NH_3 vapour	ultraviolet	bright green	bright yellow	faint purple	colourless or pale yellow	bright red	dull red
$AlCl_3$	visible	pale yellow	yellow	colourless	colourless	pale yellow	yellow
$AlCl_3$	ultraviolet	fluorescent green	fluorescent yellow	fluorescent yellow	fluorescent green	fluorescent green	fluorescent orange
Na_2CO_3	visible	bright yellow	yellow	pale green	green	orange	red
$NaBH_4$	visible	colourless	colourless	colourless	magenta	colourless	colourless
$FeCl_3$–$K_3Fe(CN)_6$	visible	blue	blue	blue	blue	blue	blue

blue and not a yellow colour (Table 1) after spraying with sodium carbonate solution. Also flavones with a methyl substituent on the 4'-hydroxyl group do not give the usual colour change in ultraviolet light on fuming with ammonia. Again, 7,8-dihydroxyflavanones are different from the others. They fluoresce bright blue on paper when fumed with ammonia in ultraviolet light[9]. Finally, as there is no specific test for isoflavones, they cannot be readily distinguished from flavans.

Elution from paper

In studying flavonoids by paper chromatography, it is necessary to have an efficient method of removing the pigments from paper for purification and further study. A useful general solvent is ethanol containing 30% water. Elution of short strips of paper containing the pigment by capillary flow from a central reservoir is best carried out in a closed vessel of the type described by GAGE AND WENDER[31]. The rate of elution is followed by removing a strip, drying it and examining it in ultraviolet light. Elution usually takes from 4 to 16 hours. With strongly adsorbed pigments the addition of acetic acid (10% by vol.) to the eluting solvent is recommended.

It is not advisable to keep flavonoids stored indefinitely on paper strips as chemical changes may take place. For example, a chalcone glycoside of *Coreopsis maritima* was converted to the corresponding aurone glycoside, when kept on Whatman No. 3 paper for several weeks[9]. The hydrolysis of glycosides on paper has been noted frequently in this laboratory.

Sources of standard markers

As already mentioned, standard markers should be run on all chromatograms. An unknown can be identified provisionally if its mixture with a sample compound does not separate when chromatographed in several solvent systems. For these purposes, samples of a reasonable number of authentic flavonoids are required. Fortunately, many of the more common flavonoids such as rutin, naringin, hesperidin and quercitrin are available commercially. Other flavonoids can easily be obtained from them by simple chemical procedures (*e.g.* acid hydrolysis, methylation, oxidation etc.). For example, quercetin can be obtained from the commercially available glycosides rutin and quercitrin by acid hydrolysis.

A number of flavonoids can be obtained from common vegetable materials. For example, "instant tea" is a source of kampferol, quercetin and myricetin[36]. Parsley seed may be extracted to obtain apiin and a hydrolysed extract contains apigenin and luteolin[37]. Roots of *Iris tectorum* and *Iris florentina*, the latter being sold as orris root in powder form, provide tectiridin and iridin respectively[4]. Medicines of vegetable origin may also be useful. Thus hyssop has been used as a source of diosmin[4].

Column chromatography

The large scale separation has been carried out on columns of silica-gel[38], magnesol (a magnesium trisilicate)[8], cellulose[7], celite[39], ion-exchange[40] or polyamide resins[41,42]. Of these absorbents, magnesol has been most used. In a typical procedure[8], a column

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6 cm in diameter and 16 cm deep is prepared from a slurry of magnesol in acetone. After washing the column with acetone, the plant extract in the same solvent is applied to it and development proceeds with water-saturated ethyl acetate. Flavonoid bands are visible on the column, when viewed in ultraviolet light. The pigments are collected in separate fractions as elution proceeds. Very strongly adsorbed pigments may have be to extruded from the column.

3. THE R_F VALUES OF FLAVONOIDS

Several hundred natural flavonoid compounds are known[12,43] and additions are regularly being made to this total. A tabulation of the R_F values of all of them cannot be made as the data available in the literature are by no means comprehensive or strictly comparable. Most of the R_F values in Tables 2–8 have been determined in this laboratory in order that they may be compared directly with each other. The R_F values of some of the compounds are given here for the first time.

On the basis of their colour reactions on paper chromatograms, the flavonoids fall into three groups. There are the flavones and flavonols which appear in ultraviolet light as intense brown to yellow spots, which may be fluorescent. Then there are the flavanones, isoflavones and related compounds which are colourless or faint purple

TABLE 2

R_F VALUES OF FLAVONE AND FLAVONOL AGLYCONES

Name	*Flavone substituents*	*R_F values in*			*Colours in ultraviolet*
		BAW	*Forestal*	*Phenol*	
Flavones					
Acacetin	5,7-diOH,4′-OMe	0.91	0.91	0.88	dark brown
Apigenin	5,7,4′-triOH	0.89	0.83	0.88	dark brown***
Diosmetin	5,7,3′-triOH,4′-OMe	0.85	0.80	0.86	dark brown
Luteolin	5,7,3′,4′-tetraOH	0.78	0.66	0.66	dark brown***
Tricin	5,7,4′-triOH,3′,5′-diOMe	0.73	0.72	0.87	dark brown***
Saponaretin	5,7,4′-triOH,8-subst.*	0.56	0.86	0.80	dark brown***
Vitexin	5,7,4′-triOH,8-subst.**	0.43	0.82	0.63	dark brown***
Flavonols					
Kampferol	3,5,7,4′-tetraOH	0.83	0.55	0.58	yellow-green
Morin	3,5,7,2′,4′-pentaOH	0.79	0.73	0.34	yellow
Isorhamnetin	3,5,7,4′-tetraOH,3′-OMe	0.74	0.53	0.66	yellow
Fisetin	3,7,3′,4′-tetraOH	0.73	0.58	0.32	yellow-green FL.†
Rhamnetin	3,5,3′,4′-tetraOH,7-OMe	0.72	0.53	0.66	yellow
Quercetin	3,5,7,3′,4′-pentaOH	0.64	0.41	0.29	yellow
Azaleatin	3,7,3′,4′-tetraOH,5-OMe	0.48	0.49	0.50	yellow FL.
Myricetin	3,5,7,3′,4′,5′-hexaOH	0.43	0.28	0.13	golden yellow
Robinetin	3,7,3′,4′,5′-pentaOH	0.40	0.36	0.18	yellow-green FL.

* $-(CHOH)_5 \cdot CH_2OH$.

** $-CHOH \cdot CH \cdot CHOH \cdot CHOH \cdot CH \cdot CH_2OH$, with a bridging O between the first and third CH.

*** These flavones become bright green or yellow-green when fumed with ammonia; the other two are unchanged.

† FL. = fluorescent.

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in ultraviolet light. Finally, there are the "anthochlor" pigments, the chalcones and aurones, which are visible in daylight as yellow spots, but which change to red when fumed with ammonia.

Flavones and flavonols

Table 2 shows the R_F values in three solvent systems of 16 flavone and flavonol aglycones. Five of them, myricetin, quercetin, kampferol, luteolin and apigenin are regularly found as glycosides in the flowers of higher plants. The three naturally occurring mono-methyl ethers of quercetin are included. Two of these, rhamnetin and isorhamnetin have very similar R_F values (see also BARBER[46]) but the third, azaleatin[47] has quite a different set of values from quercetin and the other two. The R_F values of vitexin and saponaretin are also given. These are two apigenin derivatives with hydroxylated side chains in the 8-position[48, 49]. They represent an unusual group of

TABLE 3

R_F VALUES OF FLAVONOL GLYCOSIDES

Glycoside	*R_F values in*			
	BAW	*Water*	*15% acetic acid*	*Phenol*
*Kampferol**				
3-monoglucoside (astralagin)	0.70	0.13	0.43	0.74
3-rhamnoglucoside (nicotiflorin)	0.54	0.23	0.54	0.64
3-diglucoside**	0.43	0.27	0.54	0.55
3-triglucoside**	0.31	0.33	0.51	0.45
3-rhamnodiglucoside	0.41	0.34	0.61	0.54
3-rhamnogalactosido-7-rhamnoside (robinin)	0.40	0.54	0.75	0.73
3-rhamnoglucosido-7(?)-glucoside***	0.40	0.54	0.74	0.52
7-monoglucoside	0.54	0.02	0.17	0.62
7-monorhamnoside	0.75	0.02	0.18	0.76
3-xyloglucoside	0.55	0.29	0.65	0.68
Quercetin				
3-monoarabinoside (avicularin)	0.70	0.07	0.31	0.61
3-monoxyloside (reynoutrin)	0.65	0.06	0.32	—
3-monoglucoside (isoquercitrin)	0.58	0.08	0.37	0.54
3-monogalactoside (hyperin)	0.55	0.09	0.35	0.56
3-monorhamnoside (quercitrin)	0.72	0.19	0.49	0.58
3-rhamnoglucoside (rutin)	0.45	0.23	0.51	0.46
3-diglucoside**	0.37	0.19	0.45	0.36
3-triglucoside**	0.23	0.18	0.41	0.26
3-rhamnodiglucoside	0.36	0.26	0.54	0.35
3-rhamnoglucosido-7(?)-glucoside***	0.36	0.46	0.71	0.31
7-monoglucoside (quercimeritrin)	0.37	0.00	0.07	0.40
4′-monoglucoside (spiraeoside)	0.48	0.01	0.13	0.33
3,4′-diglucoside-3′-methyl ether (dactylin)	0.38	0.27	0.62	0.63
Myricetin				
3-monoglucoside** (cannabiscitrin)	0.47	0.05	0.25	0.32
3-monorhamnoside (myricitrin)	0.60	0.15	0.44	0.39

* Colours under U.V. light with ammonia; kampferol glycosides: yellow-green; quercetin glycosides: yellow; myricetin glycosides: yellow-brown.

** Isolated from flowers of *Primula sinensis*.

*** Provisional structures for pigments isolated from flowers of *Solanum brachycarpum*.

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flavones only found so far in seven species[49] and have different R_F values from the common flavones. For example, vitexin and saponaretin (R_F's 0.06 and 0.16 respectively) are the only flavones or flavonols except morin (R_F 0.03) which are mobile on paper with water as solvent.

The R_F values of flavones not included in the list are given in the papers of BATE-SMITH[2,4], WENDER and co-workers[14,15] and GEISSMAN[17]. Of these only quercetagetin, 3,5,6,7,3′,4′-hexahydroxyflavone (R_F 0.40 in BAW, 0.29 in Forestal) has been found in several species[4]. Flavone itself, which occurs on the surface of the leaves and stems of many *Primula* species, travels near the front in most solvent systems[17]. It appears as a light-absorbing spot against a fluorescent background in ultraviolet light of 253 mμ wavelength.

The R_F values of 25 representative glycosides of the commonly occurring flavonols are shown in Table 3. Examples of glycosides with different sugars are included; they vary in number and position of substitution. In the case of quercetin, the R_F values of 5 different 3-monoglycosides, of 3-mono-, 3-di- and 3-triglucosides and 3,7- and 7-glycosides are shown. As with the anthocyanins[11], corresponding glycosides of kampferol, quercetin and myricetin can always be placed in the same order of R_F value. The approximate R_F value of an unknown glycoside in any one series may therefore be predicted with some confidence if the values for the other two members are known (see also[24]).

Table 4 shows the R_F values of 11 flavone glycosides. As with the flavonols, the number and the position of attachment of the sugar residues affects the R_F value.

TABLE 4

R_F VALUES OF FLAVONE GLYCOSIDES

Glycoside	*R_F values in*			
	BAW	*Water*	*15% acetic acid*	*Phenol*
*Apigenin**				
7-monoglucoside (cosmetin)	0.65	0.04	0.25	0.78
7-rhamnoglucoside (rhoifolin)	0.58	0.09	0.46	0.74
7-apiosylglucoside (apiin)	0.57	0.06	0.42	0.75
7-glucuronide	0.57	0.13	0.29	0.46
7-glucoside, 8-subst.** (saponarin)	0.42	0.31	0.64	0.65
4′-rhamnoside, 8-subst.*** (of vitexin)	0.50	0.46	0.69	0.57
Luteolin				
7-monoglucoside	0.44	0.01	0.15	0.56
7-apiosylglucoside	0.42	0.03	0.23	0.50
7-diglucoside	0.40	0.05	0.29	0.54
5-monoglucoside (galuteolin)	0.82	0.00	0.07	0.65
Acacetin				
7-rhamnoglucoside (linarin)	0.61	0.14	0.60	0.55

* Colours in U.V. light with ammonia: apigenin glycosides: bright green; luteolin glucosides: yellow-green; acacetin glycoside: dull purple.

** See footnote*, Table 2.

*** See footnote**, Table 2.

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Thus luteolin 7-monoglucoside and 7-diglucoside are chromatographically distinct. Also the 5- and 7-monoglucosides of luteolin have different R_F values from each other (Table 4) and from those of the 4′-monoglucoside[50] (R_F 0.68 in BAW, 0.72 in phenol, 0.34 in 15% acetic acid). The R_F values in Table 4 for luteolin 7-monoglucoside were measured on material isolated from several sources, including *Coreopsis maritima*[52], *Gesnera cardinalis*[54], *Solanum stoloniferum*[53] and *Dahlia variabilis*[54]. Another reported source of this compound, namely the leaves of *Digitalis purpurea*[51], contains two luteolin glycosides, both of which are quite different in their chromatographic behaviour from the 7-monoglucoside. One of the two compounds, for example, has an R_F of 0.30 in BAW, 0.12 in water and 0.12 in phenol saturated with water[54].

Table 5 gives the R_F values of hydroxypolymethoxy flavones derived from apigenin, luteolin, kampferol and quercetin. The use of these data in the determination of the position of sugar residues in flavones on a micro-scale[5] will be discussed later in section 7. For the separation of all the isomeric partial methyl ethers of luteolin and apigenin, NORDSTRÖM AND SWAIN[5] used buffered paper and a benzene–nitromethane–water mixture. R_F values of all the relevant methyl ethers of kampferol and quercetin

TABLE 5

R_F VALUES OF METHYLATED FLAVONES AND FLAVONOLS

Methyl derivative	*R_F values in*			*Colours in ultraviolet*	
	C_6H_6–$MeNO_2$–H_2O (3:2:5)	*n*-BuOH–H_2O		*alone*	*with NH_3*
		borate paper	*phosphate paper*		
*Apigenin**					
4′,5-dimethyl	0.46	0.51	0.62	blue	yellow
4′,7-dimethyl	0.97	0.93	0.84	brown	brown
5,7-dimethyl	0.46	0.80	0.70	blue	green-blue
5,7,4′-trimethyl	0.91	0.87	0.79	blue	blue
*Luteolin**					
3′,4′,5-trimethyl	0.35	0.26	0.34	lilac	orange-yellow
3′,4′,7-trimethyl	0.95	0.92	0.77	brown	brown
3′,5,7-trimethyl	0.72	0.67	0.55	blue	yellow-green
4′,5,7-trimethyl	0.73	0.77	0.55	blue	red-purple
3′,4′,5,7-tetramethyl	0.91	0.80	0.55	blue	blue
	BAW	*15% acetic acid*	*Forestal*		
*Kampferol***					
5,4′-dimethyl	0.81	0.04	0.84	yellow	yellow
5,7,4′-trimethyl	0.87	0.08	0.91	yellow	yellow
3,5,7,4′-tetramethyl	0.93	0.32	0.97	blue	blue
*Quercetin****					
3,5,7,3′-tetramethyl	0.83	0.19	—	blue	yellow-green
5,7,3′,4′-tetramethyl	0.75	0.05	—	yellow	deep yellow
3,5,7,3′,4′-pentamethyl	0.85	0.20	—	blue	blue

* From NORDSTRÖM AND SWAIN[5].
** From HARBORNE[54].
*** From HERMANN[55].

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TABLE 6

R_F VALUES OF FLAVANONES AND ISOFLAVONES

Flavonoid	R_F values in			
	Butanol–27% acetic acid (1:1)	BAW (4:1:5)	Water	30% acetic acid
*Flavanone aglycones**				
7,8,3′,4′-tetraOH**	0.72	—	—	0.55
7-OMe,8,3′,4′-triOH	0.76	—	—	0.63
8-OMe,7,3′,4′-triOH	0.82	—	—	0.65
7,8,4′-triOH**	0.81	—	—	0.62
6,7,3′,4′-tetraOH	0.73	—	—	0.46
7,3′,4′-triOH (butin)***	0.84	—	0.20	0.64
5,7,3′,4′-tetraOH (eriodictyol)***	0.85	—	—	0.56
5,7,4′-triOH (naringenin)***	0.69	0.89	0.16	0.66
7-OMe,5,4′-diOH (sakuranetin)	—	0.90	0.09	0.65
7,4′-diOMe,5-OH	—	0.91	0.06	0.66
5,7,3′-triOH,4′-OMe (homoeriodictyol)***	—	0.88	0.14	0.67
5,7,4′-triOH,3′-OMe (hesperitin)***	—	0.89	0.11	0.67
*Flavanone glycosides****				
Naringenin 7-rhamnoglucoside	—	0.59	0.62	0.87
Naringenin 7-glucoside	—	0.64	0.44	0.80
Sakuranetin 7-glucoside	—	0.69	0.48	0.81
Hesperitin 7-rhamnoglucoside	—	0.48	0.50	0.85
Hesperitin 7-glucoside	—	0.60	0.33	0.79
Flavanonols				
3,5,7,4′-tetraOH (dihydrokampferol)	—	0.87	0.30	0.72
3,5,7,3′,4′-pentaOH (dihydroquercetin)	0.86	0.78	0.28	0.67
Dihydrokampferol 7-glucoside***	—	0.57	0.64	0.84
Flavan				
3,5,7,3′,4′-pentaOH (*d*-catechin)	0.69	0.64	0.31	0.64
Isoflavone aglycones				
7,4′-diOH (daidzein)**	—	0.92	0.08	0.62
7-OMe,4′-OH (formononetin)**	—	0.94	0.07	0.67
7-OH,3′,4′-O_2CH_2 (ψ-baptigenin)**	—	0.94	0.07	0.66
5,7,4′-triOH (genistein)	—	0.94	0.04	0.59
7-OMe,5,4′-diOH (prunetin)	—	0.94	0.03	0.67
5,7,4′-triOH,8-OMe	—	0.91	0.11	0.70
5,7,8,4′-tetraOMe	—	0.93	0.29	0.84
5,7,4′-triOH,6-OMe (tectirigenin)	—	0.90	0.09	0.70
5,7,4′-triOH,6,3′,5′-triOMe (irigenin)	—	0.91	0.16	0.79
5,6,7,3′,4′,5′-hexaOH (irigenol)	—	0.54	0.02	0.33
Pomiferin	—	0.94	0.00	0.06
Osajin	—	0.95	0.00	0.00
Isoflavone glucosides				
Genistein 7-glucoside (genistin)	—	0.67	0.25	0.75
Tectirigenin 7-glucoside (tectiridin)	—	0.67	0.38	0.83
Irigenin 7-glucoside (iridin)	—	0.66	0.48	0.87

* From HARBORNE AND GEISSMAN[9] and HARBORNE[54].

** Colour in U.V. with ammonia: light blue.

*** Colour in U.V. with ammonia: light yellow or yellow-green (the other compounds in this table have feeble colours or are colourless under these conditions).

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are not available. The compounds that have been examined (Table 5) can be separated by the usual solvent systems[54, 55].

Flavanones and isoflavones

The R_F values of a number of flavanones, isoflavones and other related substances are shown in Table 6. These compounds have a much more restricted distribution in the plant kingdom than flavones. Most of the 16 or so naturally occurring isoflavones are included. The collection of the R_F values of flavanones is also fairly comprehensive. Water and dilute acetic acid are very useful solvent systems with these compounds.

The R_F values of two flavanonols and a common catechin are included in Table 6 for purposes of comparison. A typical monomeric flavan-3,4-diol, leucocyanidin, has R_F 0.52 in BAW, R_F 0.50 in water and other compounds of this type have very similar R_F values[25]. The positions of these latter compounds are located on paper by spraying with 3 % *p*-toluenesulphonic acid in ethanol. Red or orange yellow spots develop after heating at 103° for 5 minutes[25].

TABLE 7

R_F VALUES OF CHALCONES AND AURONES*

Structure	R_F values in				
	Butanol–27% acetic acid (1:1)**		Phenol	Water	30% acetic acid
*Chalcone aglycones****					
2′,4′,3,4-tetraOH (butein)	0.83	0.83	0.66	0.01	0.19
2′,4′,5′,3,4-pentaOH (stillopsidin)	0.65	—	—	0.01	—
2′,3′,4′,3,4-pentaOH (okanin)	—	0.56	—	—	0.08
Glucosides					
Butein 4′-glucoside (coreopsin)	0.67	0.56	0.64	0.05	0.43
Stillopsidin 4′-glucoside (stillopsin)	0.47	—	—	0.02	—
Okanin 4′-glucoside (marein)	—	0.38	—	—	0.22
Aurone aglycones					
6,3′,4′-triOH (sulphuretin)	0.87	0.80	0.70	0.01	0.19
4,6,3′,4′-tetraOH (aureusidin)	0.66	0.57	0.29	0.01	0.10
6,3′,4′-triOH, 7-OMe (leptosidin)	0.80	0.76	0.80	0.01	0.19
6,7,3′,4′-tetraOH (maritimetin)	—	0.53	—	—	0.10
Glucosides					
Sulphuretin 6-glucoside (sulphurein)	0.60	0.49	0.69	0.03	0.41
Aureusidin 6-glucoside (aureusin)	0.36	0.28	0.35	0.01	0.16
Aureusidin 4-glucoside (cernuoside)	0.56	0.49	0.45	0.02	0.25
Leptosidin 6-glucoside (leptosin)	0.55	0.51	0.73	0.03	0.33
Maritimetin 6-glucoside (maritimein)	—	0.42	—	—	0.21

* From GEISSMAN[17], GEISSMAN AND HARBORNE[57], GEISSMAN, HARBORNE AND SEIKEL[52] and HARBORNE AND GEISSMAN[9].

** In the first column R_F's were measured from front of the spots; in the second column, R_F's were measured from centre of the spots.

*** All chalcones and aurones are yellow in visible light and yellow, orange or brown in ultraviolet light.

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Chalcones and aurones

The R_F values of many of the naturally occurring chalcones and aurones[17, 52] and some of their methylated derivatives[9, 57] are collected in Tables 7 and 8. In these series, the glycosides have lower R_F values in BAW than the corresponding aglycones. The amount of this decrease in R_F value depends on the number and position of

TABLE 8

R_F VALUES OF METHYLATED CHALCONES AND AURONES

Compound	R_F values in				Colours	
	Butanol–water triphosphate paper	BAW	30% acetic acid	Butanol ammonia	U.V.	U.V./NH_3
*Butein**						
4′,3,4-trimethyl	0.86	—	—	—	brown	brown
2′,4′,4-trimethyl	0.83	—	—	—	yellow-green	green
2′,3,4-trimethyl	0.38	—	—	—	olive	yellow-green
2′,4′,3-trimethyl	0.68	—	—	—	yellow-green	yellow
2′,4′,3,4-tetramethyl	0.92	—	—	—	yellow-green	yellow-green
*Sulphuretin**						
3′,4′-dimethyl	0.25	—	—	—	yellow-green	green
4′,6-dimethyl	0.93	—	—	—	yellow	yellow-green
3′,6-dimethyl	0.54	—	—	—	yellow	orange
3′,4′,6-trimethyl	0.80	—	—	—	green	green
*Aureusidin***						
4,3′,4′-trimethyl	—	0.77	0.11	0.11	blue-green	blue-green
4,6,3′-trimethyl	—	0.77	0.11	0.33	yellow	orange
4,6,4′-trimethyl	—	0.81	0.22	0.59	blue-green	blue-green
6,3′,4′-trimethyl	—	0.79	0.14	0.13	blue-green	blue-green
4,6,3′,4′-tetramethyl	—	0.80	0.17	—	green	green
*Maritimetin****						
7,3′,4′-trimethyl	—	0.83	0.18	—	yellow-green	orange
6,3′,4′-trimethyl	—	0.83	0.20	—	green	brown
6-monomethyl	—	0.69	0.18	—	yellow	red-green
6,7,3′,4′-tetramethyl	—	0.82	0.18	—	yellow-green	yellow-green
6,7-dimethyl	—	0.78	0.19	—	yellow	red

* From NORDSTRÖM AND SWAIN[58].
** From GEISSMAN AND HARBORNE[57].
*** From HARBORNE AND GEISSMAN[9].

substitution of the sugar residues. For example sulphuretin 6-diglucoside (R_F 0.21 in BAW, 6:1:2)[58] has a lower R_F than the 6-monoglucoside (R_F 0.49; *cf.* sulphuretin R_F 0.80) and the 4- and 6-monoglucosides of aureusidin are chromatographically different (Table 7).

The R_F values in 30% acetic acid of 17 synthetic aurones have been recorded by ROUBALOVA[59].

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4. CHROMATOGRAPHIC BEHAVIOUR AND CHEMICAL STRUCTURE

The R_F values of the majority of flavonoids are directly related to their chemical structure. This correlation between chromatographic behaviour and structure was first discovered by BATE-SMITH AND WESTALL[13]. These authors plotted R_M values* in BAW against the number of hydroxyl and glucosyl substituents. They found that a straight line relationship exists between the R_M values of flavonoids and the numbers of their hydroxyl and sugar substituents. The few compounds with irregular chromatographic behaviour also have unusual structural features. ROBERTS[10, 23, 24] and his co-workers have shown that this relationship is observed with R_F values in aqueous solvents as well. SIMPSON AND GARDEN[60] and SHAW AND SIMPSON[61] have shown that the chromatographic behaviour of some simple hydroxy and methoxy flavones is dependent on their degree of chelation. ROUX AND EVELYN[25] have found that R_M value is directly related to average molecular weight in the case of tannins having flavan units in their structure.

Relationships between chromatographic behaviour and structure of known flavonoids are illustrated in Tables 2–8. The five main conclusions can be summarised as follows:

(1) The R_F values are sufficiently characteristic to make them of considerable value in the identification of flavonoids. There are many reports of the successful separation of related compounds. Two examples may be quoted, namely the separation of the optical antipodes of the tea catechins by ROBERTS AND WOOD[23] and the separation of the isomeric di- and trimethyl ethers of apigenin and luteolin by NORDSTRÖM AND SWAIN[5]. The number of pairs of flavonoids which are difficult or impossible to separate on paper are comparatively few and are, in any case, substances of very similar chemical structures. Examples are rhamnetin and isorhamnetin[46] (Table 2), quercetin and aureusidin[26] and the 3-glucoside and 3-galactoside of kampferol[24].

(2) An increase in the number of hydroxyl groups substituted in the flavonoid molecule lowers the R_F value in both alcoholic and aqueous solvent systems[10, 13]. This result is to be expected in the solvent BAW but not in water, in which solvent an increase in hydroxyl groups might be expected to raise rather than lower the R_F value[10]. ROUX AND EVELYN[25] have shown that this generalisation only applies to phenolic hydroxyl groups and additional aliphatic hydroxyl groups do, in fact, increase the R_F value in water. These authors have also pointed out that *o*-hydroxyl groups reduce the R_F value in BAW more than two hydroxyl groups which are not adjacent.

(3) Methylation of their hydroxyl groups usually increases the mobility of flavones in all solvent systems. The rise in R_F value for every hydroxyl group that is methylated is much less than the fall in R_F value caused by the introduction of a hydroxyl group[13]. As may be expected the effect on R_F value of a chelated hydroxyl group is less than

* $R_M = \log\left(\frac{1}{R_F} - 1\right)$.

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that of a free hydroxyl group. Similarly, the methylation of a chelated hydroxyl group (*e.g.* that in the 5-position) will alter the R_F value more than methylation elsewhere in the molecule (compare the R_F's of quercetin and its 5-, 7- and 3'-methyl ethers, Table 2).

(4) Glycosidation lowers the R_F value in BAW but increases it in water[10,13]. This effect applies to all classes of flavonoid, but is best illustrated by reference to the flavonol glycosides (Table 3). Increases in the number of sugar residues progressively reduce the mobility of flavonols in BAW and raise their mobility in water (compare the R_F values of 3-mono, 3-di and 3-triglucosides of kampferol and quercetin). The position of substitution of the sugar residues is an important factor in determining the R_F value of a glycoside. Kampferol and quercetin 7-monoglucosides for example have zero R_F values in water. On the other hand flavonol 3,7-triglycosides have higher R_F values in water than related 3-triglycosides.

(5) The R_F values in water of all planar flavonoid molecules are zero[10,62]; for example flavonol and flavone aglycones, chalcones, aurones and anthocyanidins. In contrast flavonoids in which the two benzene rings are not in the same plane have positive R_F values in water; *e.g.* flavan-3-ols (catechins), flavan-3,4-diols, flavanones, flavanonols, dihydro-chalcones, dihydro-aurones and most flavonol glycosides. This generalisation has been established by the observations of ROUX[62] and ROBERTS, CARTWRIGHT AND WOOD[24]. ROUX AND EVELYN[25] have also pointed out that the introduction of planarity reduces the R_F values of flavonoids in alcoholic solvents, *e.g.* BAW, or *sec.*-butanol. Thus flavanonols (*e.g.* dihydroquercetin, R_F 0.78) have higher R_F values than the corresponding flavonols (*e.g.* quercetin, R_F 0.64).

5. THE CHROMATOGRAPHIC SURVEY OF FLAVONOIDS IN PLANTS

Flavonoids are widely distributed in foodstuffs[63], have pharmacological interest (*e.g.*[64]) and are related to the natural tannins[65]. Many surveys of plant material have been carried out in order to study the taxonomic distribution of these pigments. Previous methods of detecting flavones in plants were based on simple colour tests, many of which were of doubtful validity when used on crude extracts. Chromatographic methods have the advantages that they separate the flavones from impurities, reveal mixtures of flavones and provide an accurate and rapid means of provisional identification, based on R_F values and colour reactions. The application of paper chromatography to this field has therefore been most fruitful.

In most survey work, concentrated plant extracts are applied to paper chromatograms, which are developed in one, or more frequently, two dimensions. In addition, or alternatively, acid-hydrolysed extracts are examined in the same way for the presence of aglycones. Flavonoid spots on paper chromatograms are unlikely to be confused with those of other plant pigments or other naturally occurring substances (*e.g.* simple phenols, alkaloids, etc.). They are quite distinct from those of other plant colouring matters, such as the carotenoids, chlorophylls or anthraquinones[66]. The anthocyanins are readily distinguished from flavones because of their highly character-

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istic colours in visible light. Within the group of flavonoid compounds, it is the flavones, flavonols and anthochlor pigments which are most easily detected in chromatographic surveys. The presence of flavanones and isoflavones may be missed unless special chromogenic sprays are used.

The R_F values and colour reactions of glycosides on two-dimensional chromatograms and of aglycones on a "Forestal" one-dimensional chromatogram enable the experienced worker to identify provisionally many of the flavonoids present in a plant extract. It is generally recognised that detailed chemical analysis (infrared and ultraviolet spectroscopy, melting point, etc.) is a necessary part of complete identification. Even the glycoside of a well-known flavonol, *e.g.* quercetin, cannot be identified solely on the basis of chromatographic comparison with authentic glycosides.

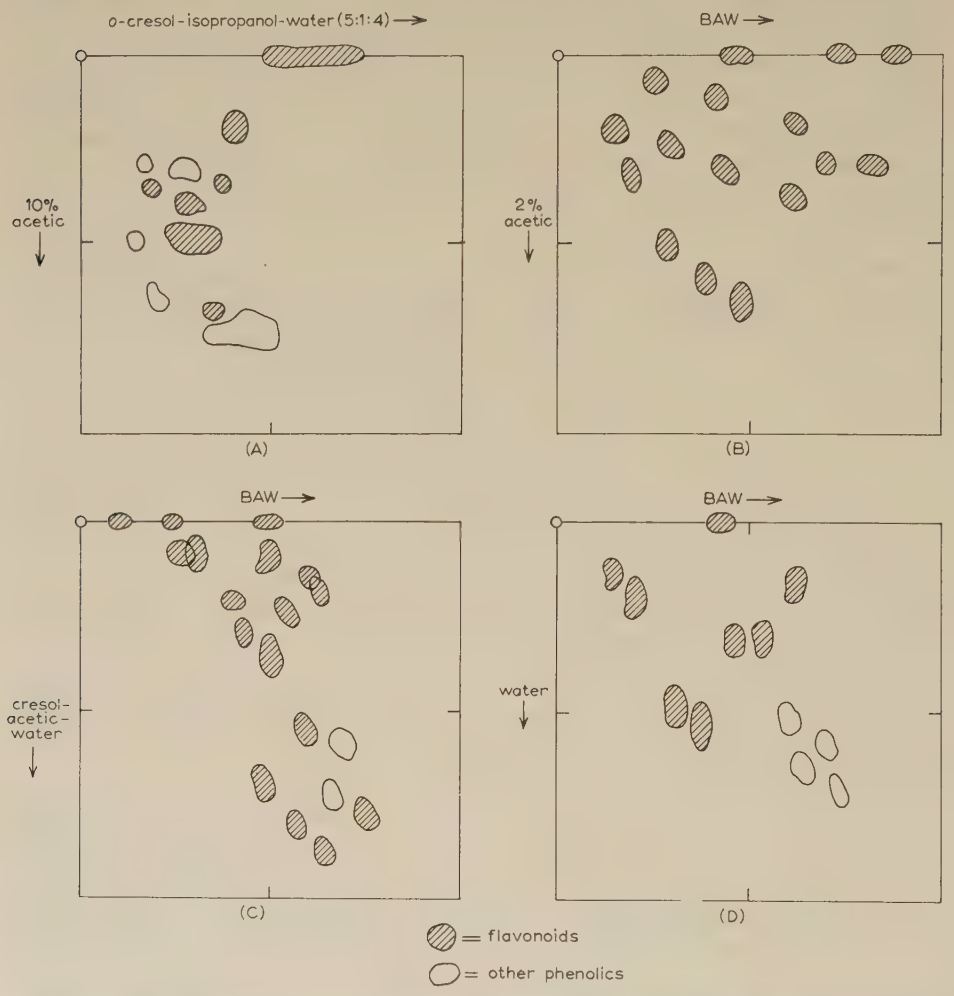

Fig. 2. Typical two-dimensional chromatograms of flavonoids present in plant extracts. (A) Leaves of *Triticum vulgare*[69], (B) Leaves of tea (flavonols only)[24], (C) Flowers of *Antirrhinum majus* (genotype PPMMYY)[6] and (D) Flowers of tuberous *Solanums*[54].

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Chromatographic surveys are nevertheless an extremely useful way in which to study the occurrence of both known and unknown substances within a particular genus or family. The distribution of flavonoids in the leaves[4] and petals[3, 44] of vascular plants has been examined by paper chromatography. In more detailed surveys of groups of related plants, the complex patterns of flavonoid spots on two-dimensional chromatograms (see Fig. 2) have been used to study these relationships. Recent surveys of the heartwood extracts of *Pinus*[67] and *Prunus*[68] species, of the leaves of *Triticum* species, allied genera[69] and *Camellia* species[70], and of the tubers and flowers of *Solanum* species[54, 71] have all been carried out by means of paper chromatography.

The detection of flavonoids by two-dimensional chromatography has played an important part in recent biochemical-genetical studies of *Antirrhinum majus*[6] and *Dahlia variabilis*[72]. In this laboratory, the occurrence of flavones related to anthocyanins has been investigated using these and other methods[45, 53, 54]. Paper chromatography is the best way of examining the petals or leaves of plant progenies for variations in their flavone content.

Another use of paper chromatography is in the preparation of medicines of vegetable origin[73]. Spurious samples of plant material may be rejected on the basis of chromatographic examination of the flavonoids and other compounds present. Finally, paper chromatography has been used to investigate the metabolic fate of flavonoids when fed to rats and humans[74, 75].

6. SEPARATION AND PURIFICATION BY CHROMATOGRAPHY

Chromatography is a well recognised method for separating flavonoids present in plant extracts. Indeed for small scale work, paper partition chromatography is the method of choice. It is simple, effective and rapid. A high degree of resolution can be achieved without much experience. On the other hand, for large scale separation, chromatography has not supplanted the time-honoured procedures of lead precipitation, fractional crystallisation or the more recent development of counter-current distribution[76]. Chromatography by adsorption on columns of magnesol or cellulose works well with simple mixtures of flavones.The method has, however, failed to separate some complex mixtures of flavonoids[5, 49, 58]. The development of new adsorbents such as polyamide resin[41] may alter this picture.

The method of separating plant flavonoids by paper chromatography is as follows. It consists simply of banding concentrated plant extracts on sheets of thick Whatman filter paper, developing with a suitable solvent (*e.g.* BAW) and excising, eluting and rechromatographing the individual bands which have separated. Further details are given elsewhere[5, 6, 9, 53]. Fig. 3 shows how the flavonol glycosides of *Primula sinensis* flowers separate on paper. The method is particularly useful in providing a means of isolating minor components and those compounds with unusual solubility properties, which might be missed in conventional isolation procedures.

Pure, crystalline flavonoids are readily obtained from plant extracts after separation on paper. There are however some plant constituents which will not

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separate on paper. NORDSTRÖM AND SWAIN[5] failed to separate naringenin and apigenin glycosides in an extract of a mauve variety[5] and chalcone and aurone glycosides in yellow varieties[58] of *Dahlia variabilis*. They assumed that their difficulties were due to the formation of "complexes" between the flavanones (or chalcones) and the other

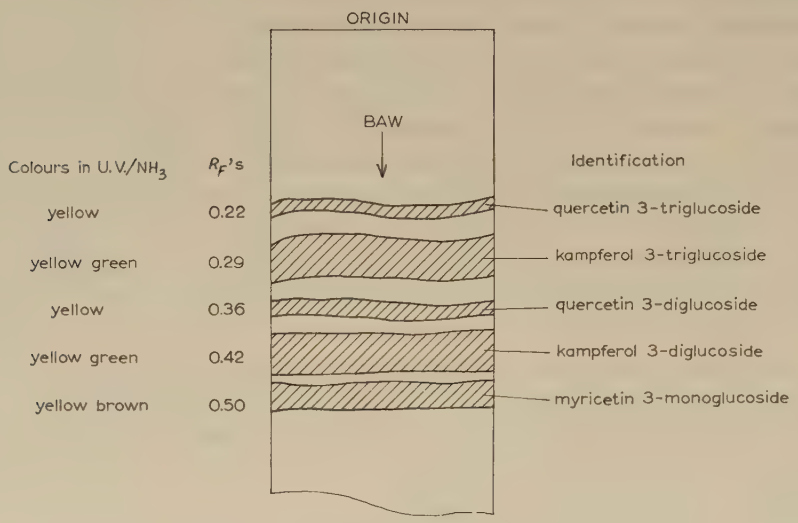

Fig. 3. Chromatographic separation of flavonols of white flowers of *Primula sinensis* (genotype "beetle") on Whatman No. 3 filter paper.

flavonoids in these plant extracts. The author has noted similar difficulties in obtaining luteolin 7-glucoside in a pure form from partially hydrolysed extracts of parsley seed[52].

Paper partition chromatography is very useful for determining the amount of separation that has been obtained by other purification procedures (*i.e.* column chromatography). It has also shown that material isolated by other extraction procedures as a single substance may contain more than one compound. For example, NYSTRÖM, HOWARD AND WENDER[77] separated two new flavonol glycosides from a supposedly pure sample of xanthorhamnin (rhamnetin 3-rhamninoside). By using 36 sheets of Whatman 3 MM paper and *n*-butanol–chloroform–acetic acid–water (4:1:1:1, by vol.) for development, they obtained 20, 80 and 10 mg of the three flavonoids from 300 mg of commercial material. In another case, a sample of "lotoflavin", which was thought to be 5,7,2′,4′-tetrahydroxy-flavone, was found on chromatographic analysis to be a mixture of kampferol and quercetin[78].

The separation of flavonoids by chromatography on magnesol was introduced and developed by GAGE, WENDER[7,8] and their associates. They isolated kampferol, quercetin and their glycosides from a number of fruit extracts (*i.e.* apricots[79], grapes[80], black-currants[81], strawberries[82] and whortleberries[83]). Other absorbents have been used for separating flavonoids. The flavones of *Spartium junceum* flowers were separated on a cellulose column by elution with water-saturated butanol by SPADA AND CAMERONI[50]. The isoflavone, biochanin A, was isolated from clover by chromatog-

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raphy on celite 545[39]. The tea catechins were separated on columns of silica gel by BRADFIELD AND PENNEY[38]. NEU[42] has separated synthetic mixtures of chalcones and flavanones on columns of polyamide resin. Finally, use has been made of chromatography on alumina or magnesol for purifying intermediates or products in chemical syntheses involving flavonoids (*e.g.*[54, 84]).

7. THE CHROMATOGRAPHIC IDENTIFICATION OF FLAVONOIDS

As mentioned in the introduction, paper chromatography plays an essential role in the identification of flavonoids on a micro-scale. The combination of this technique with that of ultraviolet spectroscopy has now been used in a large number of investigations for the elucidation of the structure of unknown compounds. The methods can be applied to as little as 1 mg of unknown substance[5] and many pigments have been identified without being isolated on a macro-scale[9]. These methods were first described by NORDSTRÖM AND SWAIN[5] and have been used and developed by numerous other investigators (*e.g.*[9, 10, 24]). The determination of the structure of flavonoid glycosides involves first the identification of the algycone, and then the determination of the nature, position and number of sugar residues. The application of paper chromatography to these two aspects will be considered separately.

Aglycone identification

In typical procedures[5, 52] the pure unknown glycoside (approx. 1 mg) is hydrolysed for 1 hour with normal mineral acid and the aglycone is extracted into ether or ethyl acetate. A portion of the concentrated extract is chromatographed and co-chromatographed with markers in at least three solvent systems (*e.g.* BAW, Forestal and phenol). A second portion is purified by paper chromatography for spectral examination[17]. All commonly found flavonoid aglycones can be distinguished satisfactorily by these methods. If the aglycone is a new one its chromatographic behaviour may suggest the way in which its structure differs from that of known compounds. Isolation of larger quantities for more detailed chemical analysis is then usually necessary. A further portion of the aglycone extract may be subjected to alkaline degradation and the simpler products (phenols and phenolic acids) identified by paper chromatographic means[78, 85]. Quercetin, for example, gives protocatechuic acid and phloroglucinol, both of which can be detected on paper, after separation in BAW, with diazotised *p*-nitroaniline[78]. The information obtained in this way is useful whether the aglycone under examination is a known or new compound.

One difficulty in this work is that some glycosides are peculiarly resistant to acid hydrolysis. At the same time, some aglycones, noticeably myricetin, are slowly destroyed by prolonged heating in acid solution. These factors may explain why some flavonoid glycosides do not give any aglycone, even after prolonged hydrolysis. Examples are a quercetin glycoside present in *Antirrhinum majus*[26], a kampferol derivative in *Freesia* flowers, apigenin glycosides of *Streptocarpus* garden hybrids[54],

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and flavone-like compounds in oil flax[86]. Other glycosides, *e.g.* those occurring in *Polygonum orientale*[87], are resistant to hydrolysis but do yield some aglycone.

Chromatographic methods are particularly applicable to the identification of flavonoid aglycones in cultivated plants. When complex mixtures of pigments occur and the analysis of individual pigments is not practicable or when single plants have to be tested for their flavonoid content, considerable reliance has to be placed on identifying the aglycone mixtures by paper chromatography. Such identifications can be confirmed by cutting out the separated aglycone spots from chromatograms,

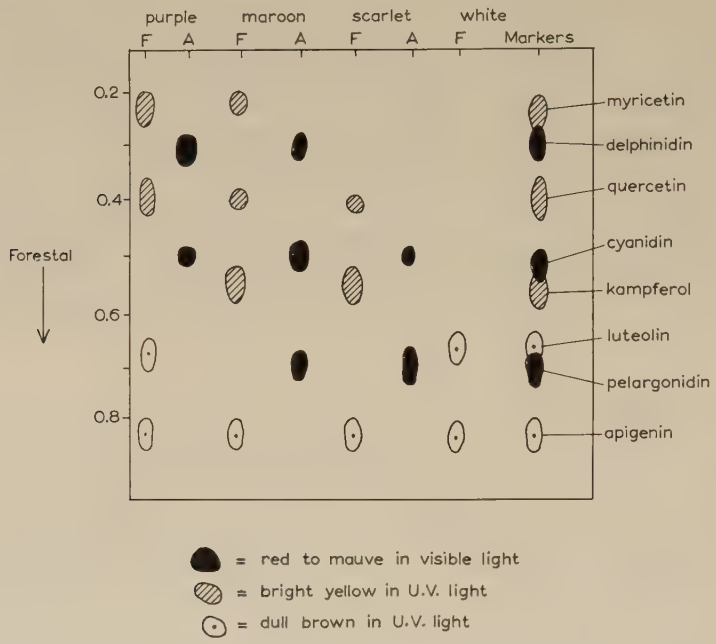

Fig. 4. One-dimensional chromatogram of flavonoid aglycones of colour mutants of *Verbena* garden hydrids; F = flavone extract (ethyl acetate), A = anthocyanidin extract (amyl alcohol).

eluting and identifying by spectral methods. Solvents containing mineral acid should be avoided in this work as BATE-SMITH[4] has found that flavonols undergo some change on chromatograms run in the Forestal solvent. Fig. 4 indicates the kind of separation that can be achieved when working with mixtures of aglycones. It shows a one-dimensional chromatogram of the hydrolysed extracts of flowers of *Verbena* garden hybrids[54]. Reference may also be made to *Antirrhinum majus*[6]. Mixtures of aglycones from this plant were separated and identified by two-dimensional chromatography.

Glycoside determination

Paper chromatography is a most valuable technique for identifying the glycosidic residues of flavonoids. Plant glycosides are often isolated in hydrated form and characterisation by classical methods (elementary analyses, m.p. etc.) may not be

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reliable. This particularly applies to glycosides containing two or more sugar residues, which may be difficult to purify by recrystallisation. The ways in which chromatography is useful in this work can be enumerated as follows.

(1) The chromatographic behaviour of an unknown glycoside, particularly in water as solvent, is a useful indication of the number and position of its sugar residues. A comparison of its R_F values with those of known glycosides of the same aglycone (see *e.g.* Tables 3 and 4) will show whether it is a new glycoside or not. One method of confirming that a particular glycoside occurs in a plant extract is to add the substance it is suspected to be to the extract. The spot of this substance on a two-dimensional chromatogram of the mixture is intensified if the two compounds are the same.

(2) The products of controlled acid hydrolysis of di- and triglycosides may be identified by paper chromatography. For example, the 3-rhamnogalactoside and 7-monorhamnoside of kampferol were formed during the hydrolysis of robinin (kampferol 3-rhamnogalactosido-7-rhamnoside)[54]. Likewise kampferol and quercetin 3-triglucosides yield the corresponding 3-diglucosides. Unlike the situation in the anthocyanin series[11], the method does not work well with flavonol 3-diglycosides. Rutin, quercetin 3-rhamnoglucoside, for example only gives traces of the 3-monoglucoside during hydrolysis[54]. On the other hand, flavone and flavanone 7-monoglucosides are easily prepared from the corresponding 7-apiosylglucosides and 7-rhamnoglucosides by partial acid hydrolysis[22, 37].

(3) The sugars produced by acid hydrolysis of flavonoid glycosides are identified by paper chromatography. Suitable procedures are described adequately elsewhere[88]. In purifying glycosides by chromatography for sugar determinations, free sugars can be removed by running a chromatogram in acetone–water (1:3), water or 15 % acetic acid.

(4) Paper chromatography may also be useful when the sugar:aglycone ratios of flavonoid glycosides have to be determined. The amounts of aglycone produced by acid hydrolysis are most easily measured by spectrophotometry. The sugars are then separated by paper chromatography, developed with aniline hydrogen phthalate, eluted separately and the concentration of colour is measured spectrophotometrically[89, 90]. Alternatively the sugars are eluted off the paper and then developed by reaction with anthrone[77]. Sugar estimations can also be made without first hydrolysing the glycoside, *i.e.* by reaction with anthrone or a resorcinol–sulphuric acid reagent[55, 91].

(5) The position of substitution of sugar residues is determined in the following way. The glycoside is fully methylated. Acid hydrolysis then produces a methylated derivative which has free hydroxyl groups in positions that were originally substituted with sugar residues[5, 9]. Paper chromatography and ultraviolet spectroscopy are used for distinguishing and identifying these derivatives on a micro-scale. The isomeric derivatives of a particular aglycone, *e.g.* apigenin (Table 5), have different colour reactions, R_F values or spectral maxima (*cf.*[5]). The requisite data are, however, still only available for a limited number of flavonoid compounds (Tables 5 and 8).

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8. CONCLUSION

The review will conclude with a summary of the advantages and disadvantages of chromatographic methods of identification. It is appropriate to do this as the value of chromatography for reducing the time and labour required for identifying unknown flavonoids is still not fully appreciated. On the other hand, the necessity for checking chromatographic identifications by means of other methods is not always realised.

The first advantage is that chromatography provides a rapid method of identification using milligram instead of gram amounts of material. Secondly, the R_F or R_M value is a new physical constant, which is comparable with the melting point as a means of identification. Thirdly, chromatography will separate the complex mixtures of pigments, which are frequently found in cultivated plants. Fourthly, it provides the first reliable method of rapidly surveying plant material for the presence of flavonoids.

The limitations of chromatography should also be borne in mind. Thus chromatography must always be used in conjunction with other appropriate analytical procedures. There is also a definite limit to its resolving power since some plant components which behave as single chromatographic entities have been shown to be mixtures. Finally, while it is of outstanding value with simple pigments, chromatography is of more limited value in the identification of flavonoids of complex structure (*e.g.* bisflavonoids, rotenoids, isoprenyl flavones, etc.).

The main future development in this field will undoubtedly be in the identification of more naturally occurring pigments, since all the flavonoid constituents have been identified in only a few plant species. Such information will increase our knowledge of the biosynthetic processes and functions of these pigments in the plant cell. For identification purposes, the measurement of R_F values of more reference compounds is needed. New solvent systems are unlikely to oust those now in use, but the discovery of some new adsorbents for column chromatography would be welcome. Finally, since chromatography can be used to detect traces of material, this technique should be of outstanding value in metabolic studies of flavonoids in plants.

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THE SEPARATION OF DIFFERENT TYPES OF HUMAN HAEMOGLOBIN

H. K. PRINS

Central Laboratory of the Netherlands Red Cross Blood Transfusion Service, Amsterdam (The Netherlands)

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I. Introduction

During the last decennium a large number of human haemoglobins have been described. Most of these haemoglobins are designated as abnormal, for they have not been found in the erythrocytes of the healthy normal human being.

Before 1949 two normal human haemoglobins were known: normal adult haemoglobin (Hb-A) and foetal haemoglobin (Hb-F). At birth, about 80% of the total haemoglobin of the infant consists of Hb-F. During the first months of life it is gradually replaced by Hb-A. In the blood of patients suffering from Cooley's anaemia, however, high percentages of Hb-F still remain during their whole life. In 1949, Pauling and coworkers[58] identified in the blood of patients suffering from the so-called "sickle-cell anaemia" a haemoglobin type differing from both Hb-A and Hb-F. The occurrence of this newly detected sickle-cell haemoglobin (Hb-S) appeared to be genetically determined. Since then, several other types of human haemoglobin have been described; these named in order of discovery are: Hb-C[34], D[35], E[37], G[14], H[65], I[69], J[81], K[9], L[1] and N[66]. A number of reviews have been written about the properties, distribution and clinical aspects of the human haemoglobins[59, 75, 5, 84, 39, 49, 50, 52, 57, 13, 29].

Apart from the clinical significance and the importance in anthropological studies, the occurrence of abnormal haemoglobins offers an interesting field of research to the biochemist. We are dealing here with a class of very closely related proteins and it is likely that many of these proteins occur as a result of a single

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mutation in one place in the system of genes responsible for the synthesis of normal human adult haemoglobin.

The family of human haemoglobins is extremely well suited for the investigation of the effect of a single mutation on protein structure. The main reason is that it is easy to obtain haemoglobin in a pure state, that is pure from the point of view of protein chemistry.

In order to study differences of protein structure between two closely related compounds it is essential that the different compounds should be identifiable.

Several methods have been used for the identification of haemoglobins. The first difference detected between Hb-A and Hb-F was the much slower denaturation-rate of the latter in alkaline solutions[44]. The so-called "alkali-denaturation method" was first used to estimate the amount of foetal haemoglobin in mixtures, and later to prepare solutions containing only foetal haemoglobin.

It was not until after the advent of electrophoresis as a general method that several other human haemoglobins were recognized, and some of them quantitatively estimated and isolated from mixtures in small amounts. Electrophoresis has since been the most generally used method for the identification and estimation of human haemoglobins. In some cases, when electrophoresis gives no satisfactory results, haemoglobins can be identified by solubility tests (differentiation between Hb-S and Hb-D).

The fact that the results obtained with nearly all the identification techniques strongly suggest that the erythrocytes of normal adults contain more than one type of haemoglobin, makes the matter more complicated. Different investigators have studied this apparent heterogeneity of normal human haemoglobin[4, 60, 20, 21, 47, 2, 24].

In this review we shall discuss some methods for the identification and estimation of human haemoglobins, and further we shall consider how far the different techniques are suitable for the isolation and purification of the different types.

Chromatographic techniques have proved to be very useful for the isolation, identification and purification of several proteins and polypeptides. Accordingly a large section of this review has been devoted to the chromatography of haemoglobins.

Techniques used for the further elucidation of the structure of a type of Hb that has already been detected, identified and isolated are not discussed here.

General properties and preparation of haemoglobin[83, 51]

The haemoglobin content of normal human adult blood is 14–16 g/100 ml. The pigment is accumulated in the erythrocytes, in which its concentration is about 32–35%. It is soluble in aqueous solutions with an ionic strength varying from 0 to very high values.

Haemoglobin is a conjugated protein with a mol. weight of about 67,000, consisting of a globin portion (96% by weight) and four "haem groups" (ferrous protoporphyrin complexes), which are readily split off in acid solution; the iron content of Hb is 0.34%.

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Each haem group in the haemoglobin molecule can bind reversibly 1 molecule of O_2 or CO; the binding with the latter is by far the strongest. The absorption maxima in visible light are characteristic for the different haemoglobin derivatives; absorption maxima for non-oxygenated haemoglobin occur at 430 and 555 mμ, for oxygenated haemoglobin (HbO_2) at 414, 541 and 576 mμ and for carbonmonoxy-haemoglobin (HbCO) at 418, 538 and 571 mμ.

On addition of potassium ferricyanide oxyhaemoglobin is readily converted into ferri-(met-)haemoglobin, in which every haem group has gained an electron and can bind anions such as chloride or cyanide, but no oxygen or carbon monoxyde. Methaemoglobin cyanide is very stable and has characteristic absorption maxima at 414 and 542 mμ.

Methaemoglobin formation also takes place in oxyhaemoglobin solutions on standing, especially at pH below 7, by auto-oxidation. This auto-oxidation can be avoided to a great extent by storage of the haemoglobin solutions in the CO-saturated state. HbCO should be used in almost all analytical or preparative work. HbO_2 is only used in the alkaline denaturation test or in some special solubility experiments.

Haemoglobin solutions are prepared as follows:

Fresh blood collected in solutions containing oxalate, citrate or heparin to prevent clotting, is centrifuged for 10 min at 600 *g*. The plasma is then pipetted off, the red cells are suspended in a 5-fold volume of 0.9% NaCl solution and the suspension is again centrifuged at 600 *g* for 10 min. This washing procedure is repeated 4 times and then the washed erythrocytes are lysed; after addition of 1 vol. of water and 0.5 vol. of toluene or octyl alcohol, the mixture is shaken vigorously for 1 min and allowed to stand overnight at 0–4° to complete haemolysis. After sharp and prolonged centrifugation (20 min, 3000 *g*) the stroma-protein (which amounts to 1% of the total protein of the erythrocyte and is the main impurity in haemoglobin solutions that have not been carefully prepared) is found between the upper (toluene) and lower layer. Toluene and stroma are pipetted off and the remaining haemoglobin solution is filtered through a thin layer of Celite 535. The resulting solution is clear and free of stroma as can be checked by adding to a sample an equal volume of saturated ammonium sulphate solution. No precipitate should appear.

Values for the haemoglobin concentration are obtained photometrically[12]; for an estimation a haemoglobin sample is first diluted and converted either into carbonmonoxy-haemoglobin (by bubbling CO gas through a solution diluted 200-fold with distilled water) or alkaline haematin (by diluting with 100 vol. of 0.1 *N* sodium hydroxide solution). The concentration of HbCO is estimated at 570 mμ, that of alkaline haematin at 640 mμ. The haemoglobin concentration of a solution prepared in this way described here, lies between 10 and 15%.

Carbonmonoxy-haemoglobin is prepared by bubbling carbon monoxide through the haemoglobin solution until a diluted aliquot no longer changes colour upon addition of $Na_2S_2O_4$. A drop of octyl alcohol is added to avoid foaming. The CO-saturated solution can be stored for some weeks at 0–4° or for much longer periods in the frozen state.

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II. Identification and Isolation Techniques

1. alkaline denaturation methods[4, 18, 50, 42, 76]

The denaturation of oxyhaemoglobin by a large excess of alkali is a pseudo-monomolecular reaction. The reaction can be followed by determining (photometrically) the concentration of either the denaturation product (at 650 mμ) or non-denatured haemoglobin (at 576 mμ) at fixed times. On plotting log percentage of non-denatured haemoglobin against time the line obtained proves to be almost straight.

When a mixture of the haemoglobins *a* and *b* is denatured, the mixture obeys the equation:

$$(C_a + C_b)_t = (C_a)_0 \cdot e^{-k_a t} + (C_b)_0 \cdot e^{-k_b t}$$

If one velocity constant is large compared with the other, one term will become negligible within a few minutes and the semi-logarithmic plot becomes a straight line. Extrapolation of this line to zero time gives the percentual amount of the component with the lower denaturation velocity in the original mixture.

This method can only be applied for the estimation of foetal haemoglobin for this is the only alkali-resistant human haemoglobin known so far. The most precise estimation of low percentages of foetal haemoglobin in mixtures can be made by following the decrease of non-denatured haemoglobin at 576 mμ according to the modification of Jonxis and Visser[42]:

0.1 ml of oxyhaemoglobin solution or blood is diluted with 10 ml of water to which 2 drops of 10% ammonia are added. The extinction of this solution is measured at 576 mμ (E_b). 0.1 ml of the same haemoglobin solution or blood is diluted with 10 ml of 0.06 *N* sodium hydroxide solution, containing 2 drops of ammonia 10%. After homogenizing, the extinction at 576 mμ (E_t) is measured every minute during a period of about 15 min. After that the solution is placed in a water bath at 37° for 15 min, cooled again to room temperature and the extinction (E_e) is measured. The percentage of non-denatured haemoglobin at time *t* can be calculated with the equation:

$$\text{percentage non-denatured } HbO_2 = \frac{E_t - E_e}{E_b - E_e} \times 100$$

With this method Hb-F can be detected, when it is present in amounts above 1% of the total haemoglobin. When more than 5% is present it can be estimated with accuracy. Jonxis and Visser point out that the method is only suitable for laboratories with an accurate spectrophotometer, for the light absorption maximum of oxyhaemoglobin at 576 mμ is very sharp.

In Singer's precipitation technique[76], measurements can be carried out with a less accurate photometer:

One minute after the addition of 0.1 ml of an approximately 10% HbO_2 solution to 1.6 ml *N*/12 NaOH solution the denaturation reaction is stopped and simultaneously the denatured haemoglobin is salted out by the addition of 3.4 ml of a half-saturated ammonium sulphate solution containing 1 ml 10 *N* HCl/400 ml. The haemoglobin, remaining in solution is measured photometrically at 540 mμ.

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The SINGER method gives values that are too high at very low percentages of Hb-F and too low at high percentages[41]. SINGER attributes too high "one-minute values" at low Hb-F concentrations to the presence of soluble degradation products of denatured Hb.

The precipitation method is of great advantage for preparative purposes[10,72]. If a solution of alkali-resistant haemoglobin is contaminated with alkali-labile Hb, this alkali-labile fraction can be removed almost completely by making the solution alkaline for a few minutes:

To 2 vol. of a 10% HbO_2 solution 4½ vol. of 1/16 *N* KOH solution are added. The alkali-labile fraction is completely denatured and the alkali-resistant fraction only partly. After 2 min the solution is mixed with 16 vol. of a saturated ammonium sulphate solution, to which 0.95 ml HCl (sp. gr. 1.19) per 100 ml has been added. Denaturation ceases and the denatured Hb is salted out. The remaining haemoglobin consists to an extent of 100% of the alkali-resistant component.

Solutions of 100% Hb-F are prepared in this way from cord blood haemolysates[72].

The foetal haemoglobin, though not salted out, does not remain unaltered; it no longer crystallizes and is digested more rapidly by enzymes than native Hb-F.

It is clear that there are not many cases where the alkaline denaturation technique can be applied although it is the first method used to differentiate between two human haemoglobins and between haemoglobins of different species. Hb-F is the only human haemoglobin that can be detected, estimated and prepared by this method.

2. ELECTROPHORESIS

Moving-boundary method

As in other branches of protein chemistry, the so-called "moving-boundary method" of Tiselius has proved to be very advantageous in the field of haemoglobin research. The subject has been reviewed by experienced workers, such as ITANO *et al.*[39], so that only a brief summary of the most important features will be given here.

Good separations of different haemoglobin types can be obtained by application of the Tiselius method to 1% HbO_2 or HbCO solutions in cacodylate and phosphate buffers (pH 6.5, ionic strength 0.1) or in barbital buffer of pH 8.6 (ionic strength 0.06). In special cases buffers of very low ionic strength (0.01) are also useful.

Generally a potential gradient of 6–8 V/cm is applied for a period of 6 hours. Fig. 1, taken from ITANO *et al.*, gives a good illustration of the possibilities and the limitations of the moving-boundary technique in the analysis of haemoglobin mixtures. Good separations are obtained in many naturally occurring mixtures, such as Hb-A + S, A + D, A + E, F + E, F + S, A + C separate. On the other hand, Hb-S and C separate rather poorly, special buffer solutions[3,71] are required to differentiate between Hb-A and F and Hb-S and D are not separable by the moving-boundary technique.

Some electrophoretical data of different haemoglobin types are listed in Table 1.

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TABLE 1

ELECTROPHORETICAL CHARACTERISTICS OF DIFFERENT TYPES OF HUMAN CARBONMONOXY-HAEMOGLOBIN

	Hb type											
	H	*I*	*J*	*K*	*A*	*F*	*L*	*G*	*S*	*D*	*E*	*C*
Iso-electric point of HbCO in phosphate buffer, ionic strength 0.1	6				6.87	6.98		6.98	7.09	7.09	7.20	7.30
Mobility in phosphate buffer pH 6.5, ionic strength 0.1 ($\times 10^{-5}$ cm^2 V^{-1} sec^{-1})	— 1.5	1.7	2.0	= A	2.4	2.4	= A	≤ S	2.9	2.9	between A and S	3.2
Range of mobilities in barbital buffer, pH 8.6	H >	I >	J >	K >	A >	F >	L >	G >	S =	D >	E >	C

From the table it can be seen that some pH–mobility curves, for instance those of Hb-E and Hb-S cross each other. Therefore it is advisable to identify an abnormal haemoglobin type at not less than two pH-values.

In some cases, quantitative results are obtainable. As ITANO *et al.*[39] pointed out, percentages in unknown samples are best established by comparing with known artificial mixtures.

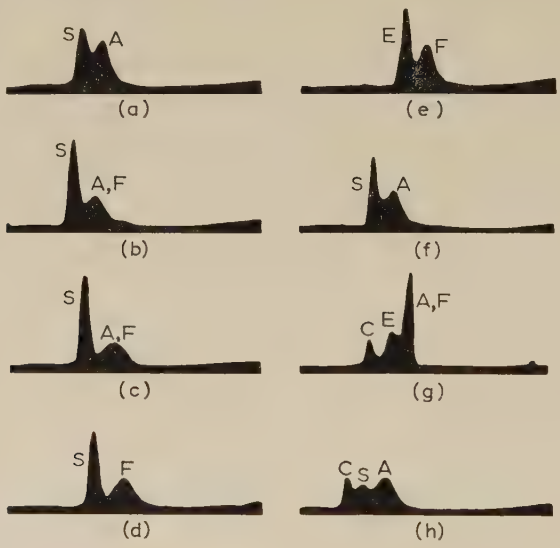

Fig. 1. Moving-boundary electrophoresis diagrams of human haemoglobin mixtures. Ascending boundaries; direction of migration from right to left; cacodylate buffer pH 6.5 and ionic strength 0.1. (ITANO *et al.*[39]). (a) sickle-cell trait; (b) sickle-cell anaemia, after transfusion of normal blood; (c) sickle-cell thalassaemia; (d) sickle-cell anaemia with about 40% foetal Hb; (e) Hb-E thalassaemia with about 40% foetal Hb; (f) artificial mixture of Hb-A and Hb-S, with about 40% Hb-A; (g) artificial mixture of Hb-C trait and Hb-E thalassaemia; (h) artificial mixture of Hb-C trait and sickle-cell anaemia.

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Although the moving-boundary method is very important in mobility studies (especially at acid pH, as will be pointed out later) and in the determination of isoelectric points, it is not suitable for routine analysis. The (expensive) apparatus requires highly trained personnel and only a few analyses can be carried out in a day.

Zone electrophoresis

Zone electrophoresis, especially on paper strips or sheets, requires a much less complicated apparatus than free electrophoresis. Up to now, paper electrophoresis has been by far the most frequently applied routine method for the identification and semi-quantitative estimation of human haemoglobins. It has the advantage that on one sheet or strip of paper a number of haemoglobin samples can be run simultaneously; for one analysis, only a few tenths of a milligram of haemoglobin are required.

Paper electropherograms can be made in several ways: in one type of apparatus the sample runs on an "open" horizontal or vertical strip or sheet, from which evaporation is possible during electrophoresis[56]. In other types of apparatus, the so-called "pressure plate" or "closed plate" systems according to KUNKEL AND TISELIUS[46], a fairly thick paper sheet is pressed between two glass plates; in this way evaporation is prevented. We obtained results of excellent reproducibility[25,62] with the closed plate method according to SMITH AND CONLEY[77]. The procedure was as follows:

The apparatus consisted of a normal tank for horizontal paper electrophoresis, in which the paper sheet (Whatman No. 3 MM, 45 × 13 cm) is pressed between two siliconized glass plates (30 × 15 × 1 cm) by means of metal clamps. The buffer used is a barbital–sodium barbitalate solution pH 8.6, ionic strength 0.06 (composition: 10.3 g sodium barbitalate + 1.4 g barbital per l of distilled water). In the middle of the dry paper sheet a pencil line is drawn across the sheet. On this line (the starting line) a series of equidistant spots (the sites of application of the samples to be run) are marked. After the sheet has been soaked in the buffer solution it is blotted between two sheets of ordinary filter paper. Then the different haemoglobin samples are brought onto the paper (0.001 ml of a 10% HbCO solution for each spot), the sheet is clamped between the two glass plates, and the two ends of the sheet are hung in the buffer compartments of the electrophoresis tank. A potential of 350 V is applied to the platinum electrodes (the potential gradient in the paper is then approximately 10 V/cm). Good separations are generally obtained after a run of 8 h. In the apparatus described here, HbCO-A migrates in this time a distance of about 5 ½ to 6 cm towards the anode, sickle-cell haemoglobin (HbCO-S) migrates in the same time about 4 cm in the same direction. After completion of the run, the paper is dried in a hot air oven at 130° for 15 min and then stained in a solution of 0.4 g Amido Black in a mixture of 900 ml methanol and 100 ml acetic acid. After staining for 15 min, the paper is washed with a methanol–acetic acid mixture (also 9:1) until the background colour is faintly blue.

Under these conditions staining is not necessary for the identification of the haemoglobin sample, but it is very important for the conservation of the pattern. A semi-quantitative evaluation of the pattern can also be obtained with the stained

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sheet; the dye then is eluted from the spot in a few ml of a mixture of 50 ml of a 10% sodium carbonate solution and 50 ml of methanol.

Estimations by this method are, however, not very reliable. Much of the protein is adsorbed by the paper when the haemoglobin spot moves across it. This "trailing" caused by adsorption has always been the main disadvantage of paper electrophoresis.

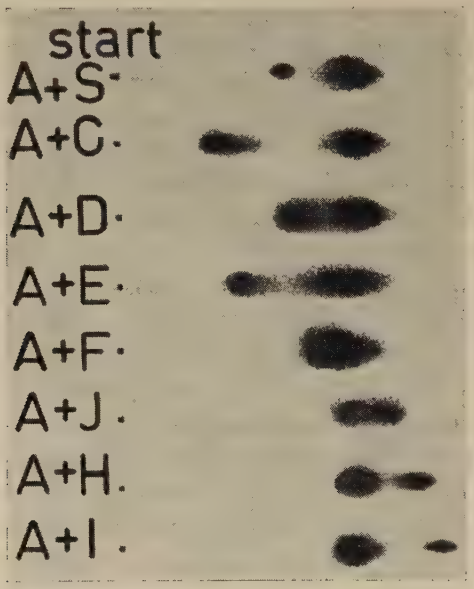

Fig. 2. A closed plate paper electrophoretic pattern of various mixtures of Hb-A with other human haemoglobin types. Whatman paper No. 3 MM; barbital buffer pH 8.6, ionic strength 0.06.

Therefore, we consider this method as very useful only for the qualitative analysis of haemoglobin mixtures. Separations obtainable with paper electrophoresis are illustrated in Fig. 2, in which patterns of mixtures of Hb-A with various other human haemoglobins are presented. Fig. 2. also gives a good impression of the practical utility of paper electrophoresis in the study of haemoglobinopathies.

The haemoglobins S, C, D, E, H, I, J can very easily be distinguished from Hb-A. On the other hand, resolution of mixtures of Hb-A with Hb-F or Hb-K, is rather difficult; Hb-D and Hb-S are indistinguishable at any pH and Hb-H and Hb-I only show a difference at acid pH. In order to obtain a good identification it is therefore necessary, as in the moving-boundary technique, to produce electrophoretic patterns at both acid and alkaline pH. However, good electrophoretic separations at acid pH can hardly be obtained because of the decreased stability of Hb at acid pH and the increased adsorption of Hb on paper.

Summing up the main features of paper electrophoresis in Hb determination, we can say that paper electrophoresis is a very simple, rapid and inexpensive routine

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method for the detection of most of the abnormal haemoglobins. However, not all haemoglobins are easily distinguished with this method and it is only suitable for working at alkaline pH.

Separations are disturbed by trailing, caused by adsorption; therefore in complex mixtures only the main components can be detected and quantitative results are difficult to obtain. Mobility studies are impossible because of the electro-osmotic flow, evaporation flow and the inhomogeneity of the electric field in the paper.

KOHN[45] introduced a *cellulose acetate strip* in zone electrophoresis, by which means adsorption was overcome to a great extent. His application of these cellulose acetate strips in electrophoresis of serum proteins and also of haemoglobins resulted in very clearly separated protein zones, not disturbed by trailing. We adopted KOHN'S technique and found in preliminary experiments that his results were very well reproducible. A short account of our modification of KOHN'S method is given here. The technique is an example of the "open strip" method.

The apparatus is shown in Fig. 3. The strips (5 × 10 cm) are stored in a cold barbital buffer solution, pH 8.6 (see p. 135), ionic strength 0.04. The same buffer is also used in the electrophoretic tank. Before use the strips are blotted with ordinary filter paper, placed in the tank and then a current of 2½ mA per strip is passed for half an hour before applying the sample. The haemoglobin samples can be applied in two ways: in spots (0.001 ml of a 10% HbCO soln./spot) or in lines (0.005 ml/line). The samples are applied 2½ cm from the anode end of the strip. After application of the samples, the same current as before is passed through for 3 h. Evaporation and electro-osmosis cause haemoglobin to migrate towards the cathode. The drying and staining procedures are the same as described on p. 135, except that the drying, staining and washing periods are all reduced three times. Before scanning, the blotted strips can be cleared by immersion in glycerol.

Results obtained in preliminary experiments with this technique are shown in Fig. 4. It can be seen, that the patterns show more detail than paper strip patterns; for instance in normal human adult haemoglobin the so-called A_2 fraction, which will be discussed later (p. 139), is clearly visible and can be measured to a certain extent. Scanning diagrams are easily obtained and do not indicate any trailing.

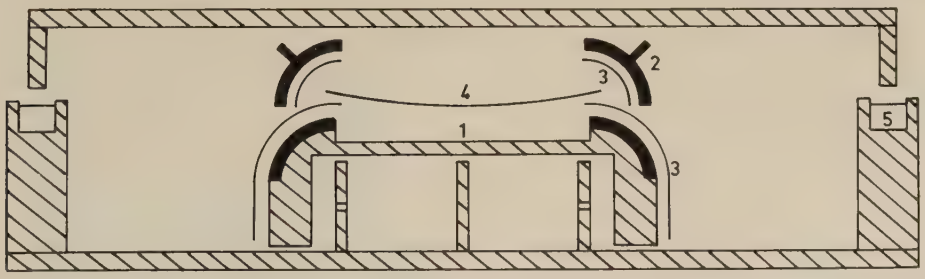

Fig. 3. Diagram of an electrophoretic tank, according to KOHN. Material: Lucite (Perspex). 1 = bridge; 2 = strip-holder; 3 = filter paper; 4 = strip; 5 = water. This apparatus is also suitable for strips of 5 × 20 cm.

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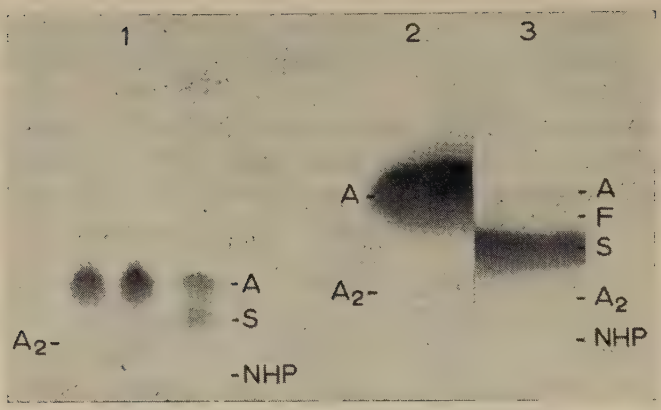

Fig. 4. Electrophoresis of various haemoglobin mixtures on cellulose acetate strips. Barbital buffer pH 8.6, ionic strength 0.04. 1. Spots of Hb-mixtures, run simultaneously on one strip. 2. Normal adult haemoglobin. 3. Haemoglobin in heterozygous sickle-cell-thalassaemia disease. N.H.P. = Non-haem protein.

The following disadvantages may be mentioned:

(1) The strips are very thin (thickness 13 μ) and therefore sensitive to evaporation.

(2) The strip material is brittle in the dry state, which makes it more difficult to handle than paper.

We expect that in spite of these disadvantages the method will prove to be very useful in work on haemoglobins.

Preparative zone electrophoresis

Many methods have been described for the separation of proteins on a preparative scale, use being made of differences in iso-electric points and electrophoretic mobilities. A comprehensive review on this subject has been given by SVENSSON[80] in 1949. Since then, great progress has been made in preparative zone electrophoresis in thick layers[48] or in columns of a zone-stabilizing medium, for instance agar, starch grain, starch gel or esterified cellulose. Most of the work on haemoglobins is carried out by starch-grain electrophoresis, following the procedure of KUNKEL AND SLATER described below, or modifications of it[48]:

Potato starch is washed several times with barbital buffer solution pH 8.6, ionic strength 0.05, by repeated stirring, settling and decanting. Horizontal starch blocks are then prepared by pouring a very thick suspension of the starch into moulds of wax paper or plastic trays of various sizes. After the starch has settled, the excess buffer solution is removed with blotting paper and the surface is smoothed. A narrow slit is made across the block and the dialysed haemoglobin solution, either pure or mixed with starch is introduced. A glass or plastic plate is pressed on the surface of the block, and after equilibrating in a cold room, a current is passed through it over a long period. Generally, the starch block is connected with two large electrode vessels by thick blotting paper or cloth covered with cellophane or wax paper. After the run, the different fractions are cut out and eluted from the starch.

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Good results are also obtained with a simple form of column electrophoresis[63]. In our laboratory we use starch grain columns of 40 cm length and 2 ½ cm diameter (Fig. 5). Columns are prepared by pouring a dilute suspension of the washed starch in barbital buffer (pH 8.6, ionic strength 0.03) into a cylindrical glass tube with a sintered glass disc at the lower end. The starch is allowed to settle and when most of the excess of buffer has flowed through the column, more of the starch suspension is introduced. This procedure is repeated until, after settling, the desired column height has been reached. During an equilibration time of a day, care should be taken that there is always some buffer solution above the column surface. Before starting an experiment, the excess of buffer is allowed to drain into the column; 2 ml of a dialysed 10% HbCO solution is carefully applied and washed as a zone of approximately 2 cm width to the middle of the column. Then the upper end of the column is connected with the anode vessel (Fig. 5) and a current of 10 mA is applied during 48 h. After completion of the electrophoretic run the column is placed above a fraction collector and the different zones are eluted from the column.

Starch grain electrophoresis is used for analytical as well as preparative purposes.

Very remarkable results have been obtained by KUNKEL AND WALLENIUS, who employed horizontal starch-slab electrophoresis[47] using barbital buffer, pH 8.6, ionic strength 0.05–0.1 (Fig. 6). The procedure permitted the electrophoretic separation of the main fraction of Hb-A from both a slower and a faster moving coloured component, regardless of whether HbO_2, HbCO or Hb was employed. It was possible to isolate these components by electrophoretic runs in which 15 ml of a dialysed 8% HbCO solution was applied in long slits. The slower component proved to be identical with the main fraction as regards visible and U.V. light absorption, as well as sedimentation constant. Both main and minor fractions, once purified, behaved as single

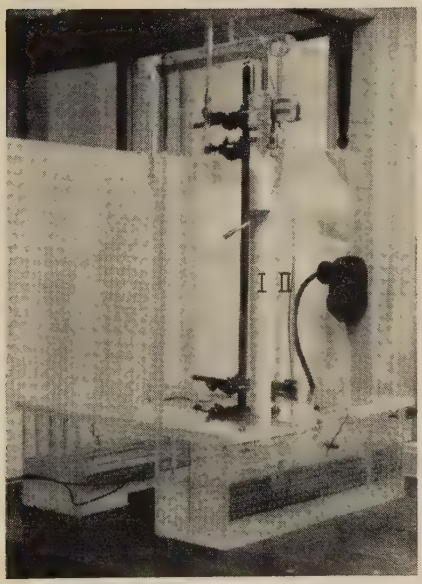

Fig. 5. Assembly for the column electrophoresis of haemoglobin samples. In one column (I) a haemoglobin sample is running; the other glass tube (II) is empty.

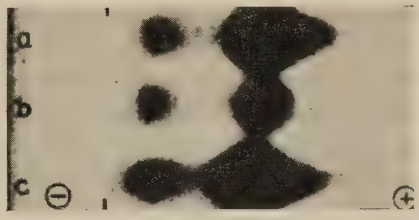

Fig. 6. Starch-grain electrophoresis of haemolysates of normal red cells (a and c) and E-trait cells (b). Barbital buffer pH 8.6; ionic strength 0.05. The lines indicate the site of application (KUNKEL AND WALLENIUS[47]).

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components in repeated electrophoretic experiments. The slow fraction resembled Hb-E very closely in starch electrophoresis, and also in moving-boundary electrophoresis at pH's 8.6 and 6.5. Quantitative analyses of a number of blood samples of adults indicated that the mean value of the amount of the slower fraction was 2.6% of the total haemoglobin, with a range of 1.8 to 3.5%. Increased amounts were found in patients with thalassaemia minor.

The findings of KUNKEL AND WALLENIUS have been confirmed by various groups of investigators.

GERALD AND DIAMOND[16], investigating the blood of parents of patients with thalassaemia major, found an average value of 4.7% for the Hb-E-like fraction (designated A_2 fraction: $\sigma = \sqrt{\frac{\Sigma d^2}{n}} = 0.8\ \%$). In 200 normal healthy human adults MASRI, JOSEPHSON *et al.*[53] found an average of 2.55% Hb-A_2 ($\sigma = 0.62\%$) and in patients with thalassaemia minor 6.49% ($\sigma = 1.5\%$). Like KUNKEL AND WALLENIUS, they found a very low amount of Hb-A_2 in cord blood (0.88%, $\sigma = 0.15\%$). As the various reported investigations indicate, starch-block electrophoresis can be very helpful in the diagnosis of thalassaemia minor, although great caution is advisable in drawing conclusions, for some other haematologic disorders are also apt to produce abnormal A_2 values[53]. A disadvantage of both the starch block and starch column methods is that they are laborious, and therefore not well suited for routine analysis. Perhaps in future the cellulose acetate strip will replace the starch slab for analytical purposes[45].

As a preparative method, zone electrophoresis has been found to be very advantageous for the isolation and characterisation of some haemoglobins and haemoglobin fractions, for instance for the preparation of "electrophoretically pure" Hb-A[64], Hb-H[23] and Hb-A_2[2], for amino acid composition analyses and terminal amino acid determination.

3. SOLUBILITY

One of the first observed reactions indicating that some abnormal haemoglobins might have deviating solubilities, is the so-called "sickle-cell reaction". Erythrocytes of patients with sickle-cell disease or "trait" lose their normal biconcave shape in oxygen-poor suspensions and change into sickle-shaped particles. After PAULING and coworkers[58] had found that these sickling cells contain an abnormal haemoglobin (Hb-S), it was believed that the sickling phenomenon is caused by the formation of highly ordered structures of the abnormal haemoglobin in the reduced state. Viscosimetric studies[2a] have revealed that in the deoxygenated form Hb-S can form large, birefringent aggregates, not only by itself but also with normal adult Hb and Hb-C. This tendency to aggregation is reflected in a markedly reduced solubility of deoxygenated Hb-S in various solutions.

The solubility of Hb-S and various other human haemoglobins were extensively studied by different investigators, employing the "variable solvent method" of ROCHE, DERRIEN and co-workers[67, 68].

In this method a constant volume of a Hb solution is added to each of a series of

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dilutions of 3.5 *M* phosphate solution, pH 6.5. After several hours of equilibration at 20°, the mixtures are filtered, and the extinction of the filtrates at 542 μ is measured and plotted against the phosphate concentrations of the phosphate–Hb mixtures.

Some examples of the solubility curves thus obtained are presented in Fig. 7 (taken from HUISMAN *et al.*[27,73]). The curves of red cell haemolysates from blood of normal adults, from cord blood, and from blood of patients homozygous for the

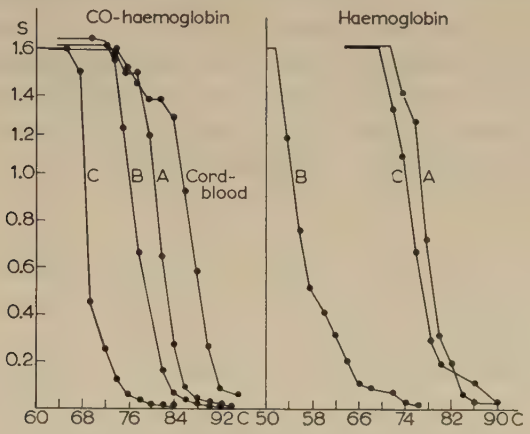

Fig. 7. Solubility curves of different human haemoglobins (HUISMAN *et al.*[27]). Abscissa: Percentage (by volume) of a 3.5 *M* potassium phosphate solution pH 6.5. Ordinate: Extinction at 543 mμ of the Hb solution after filtration.

Hb-S and for the Hb-C gene are given. It is evident from the curves that there are significant differences between the various types of haemoglobin.

The solubility curves of normal adult haemoglobin and cord blood haemoglobin obtained by ROCHE, DERRIEN and co-workers[68], strongly suggest that foetal as well as normal adult haemoglobin are not homogeneous.

ROCHE *et al.*[68] conclude from their experiments that cord blood contains, besides a low percentage of adult haemoglobin, different types of foetal haemoglobin, and that red cell haemolysates from normal human adults contain more than one type of haemoglobin, at least one of these components being of a foetal type. However, percentages of foetal haemoglobin, as large as measured by ROCHE *et al.*, have never been found in normal human adult blood by other techniques such as alkaline denaturation or an immunological method devised by CHERNOFF[10]. Partial alkaline denaturation of normal adult red cell haemolysate and estimation of the amino acid composition of the alkali-resistant part carried out by HUISMAN *et al.*[28] indicated that less than 1% of total Hb is Hb-F. Apart from differences of opinion about the explanation of solubility curves, the variable solvent method of ROCHE, DERRIEN *et al.* is a very helpful technique in the characterisation of any newly discovered haemoglobin type. For routine analyses it is unsuitable because it is too laborious. In many cases, the estimation of one or two points of a solubility curve, as in ITANO's simple modification[36], is already very helpful, for instance in differentiating between Hb-S

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(low solubility in the deoxygenated state) and Hb-D (solubility similar to that of normal adult Hb):

8 ml of a solution containing 281.2 g (1.62 mol.) K_2HPO_4 and 160.6 g (1.18 mol.) KH_2PO_4 per l, are placed in a 10 ml volumetric flask into which 100 mg of $Na_2S_2O_4$ have been weighed. After dissolution of the hydrosulphite, 1 ml of water is layered over this solution, then a solution of 50 mg HbO_2 is added, the flask is immersed in a 25° water bath, the volume brought to exactly 10 ml and finally the contents are mixed. The final phosphate concentration is 2.24 *M*. After appearance of a solid phase, the mixture is centrifuged at high speed or filtered. The amount of haemoglobin remaining in solution is measured photometrically. Under the conditions described, Hb-D remains completely in solution, whereas Hb-S is largely salted out. The solubility in 2.58 *M* phosphate is estimated by starting with 9.20 ml of phosphate solution instead of 8 ml.

4. COLUMN CHROMATOGRAPHY

Chromatography has some advantages in common with zone electrophoresis:

(1) The technique is simple.

(2) The procedure can be easily converted from an analytical to a preparative scale.

(3) A series of analyses can be carried out simultaneously (more than one column can be placed above a time-operating fraction collector).

A combination of chromatography and zone electrophoresis has been used with great success in many biochemical investigations, for instance in the elucidation of insulin structure[70].

Protein chromatography differs in many respects from the chromatography of smaller molecules. The limited stability of proteins is important in this connection. In general, proteins are only stable in aqueous solution, in definite pH and salt concentration regions. Moreover, the denaturation rates increase steeply with rising temperature. These facts have the following consequences:

(a) Elution with organic solvents is in most cases impossible. One is restricted to the use of buffer solutions, the eluting properties of which can be controlled by varying the pH and salt concentration.

(b) Application of partition chromatography, apart from a few exceptions[79], is out of the question. In particular haemoglobin becomes denatured or decomposes readily in contact with organic solvents. Paper chromatography of haemoglobin with acetic acid–pyridine mixtures[4] results in haem being split off[63].

(c) Cooling during chromatography is advisable and sometimes necessary.

When the conditions necessary for obtaining a stable solution of a given protein have been fulfilled, this does not always mean that the protein can be chromatographed under these conditions without denaturation, for chromatography itself sometimes causes denaturation. During strong adsorption, great and irreversible changes may occur in the spatial structure of the protein molecules; elution then yields solutions of non-native, that is denatured, protein.

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In general, weak adsorption is reversible. Furthermore, weak adsorption has the advantage that the eluted compounds are obtained in relatively high concentrations and that R_F values are high and therefore the time of operation short, which is important in work with material susceptible to thermal denaturation.

When selecting a suitable adsorbent for protein chromatography, ion-exchange adsorbents are apt to be preferred, for as far as elution fluids are concerned, only aqueous systems (buffer solutions) can be used.

Another requirement in protein adsorption is that the adsorbent should be of relatively small particle size. There are two reasons for this. The first is that protein molecules owing to their large dimensions cannot enter the framework of, for instance, synthetic ion-exchange resins, and therefore adsorption is restricted to the particle surface. To ensure good adsorption this surface has to be large. In the second place, because of the large dimensions of the protein molecules, the diffusion velocity from a certain place in the solution to the adsorbing surface is low. Therefore the path of diffusion must be as short as possible, in order to attain adsorption equilibrium rapidly. For this reason the liquid channels between the particles should be narrow and that, in turn, makes small particle size necessary.

Very good results have been obtained in haemoglobin chromatography by using carboxylic cation exchangers such as fine-grain Amberlite IRC-50 and carboxymethyl cellulose. These results will be discussed in the next sections.

Adsorption on Amberlite IRC-50

Amberlite IRC-50 (provided by the Rohm and Haas Company, Philadelphia), a copolymer of 95% methacrylic acid and 5% divinylbenzene, contains 9–10 mequiv. COOH/g dry weight. In work with proteins a fine mesh grade (XE-64 or 97) of this resin has been used extensively with great success (see the review by MOORE AND STEIN[54]). To understand the nature of the adsorption of a protein molecule on a weakly acid substance, such as Amberlite IRC-50, it is better to start, as BOARDMAN AND PARTRIDGE[8] did, by considering the pure ion-exchange adsorption of a small molecule such as a basic amino acid.

In this ion-exchange adsorption three equilibria are important:

$$R{-}NH_2 + H^+ \rightleftarrows R{-}NH_3^+ \quad (1)$$

$${-}COONa + R{-}NH_3^+ \rightleftarrows [COO^-]\,[R{-}NH_3^+] + Na^+ \quad (2)$$

$${-}COONa + H^+ \rightleftarrows {-}COOH + Na^+ \quad (3)$$

Although a high percentage of the amino acid will be in the cationic form at low pH, the ion-exchange resin will be almost completely in the COOH form, so that no ion-exchange adsorption will occur. At a high pH value most of the resin is in the COONa form, but now the amino acid is no longer in the cationic state, so that again no adsorption takes place. Nearly neutral conditions will be optimal for ion-exchange adsorption, for then the resin can be in the sodium form to a considerable extent and the greater part of the basic amino acid will be in the cationic form.

Experiments carried out by BOARDMAN AND PARTRIDGE[8] on the adsorption of

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lysine (iso-electric point 9.7) on Amberlite IRC-50 were in good agreement with the theoretical considerations. Around pH 7 there was maximal adsorption of lysine from buffer solutions containing 0.34 g.ions Na^+/l. Adsorption decreased gradually towards lower pH values and more abruptly at higher pH. Extending ion-exchange adsorption theory to poly-ions such as basic polypeptides and proteins one would expect qualitatively the same adsorption behaviour. Equation (2) becomes:

$$z\,Na_S^+ + P_L^{+n} \rightleftarrows P_S^{+n} + z\,Na_L^+ \tag{2a}$$

in which z is the number of sodium ions exchanged by the adsorption of one poly-ion. If $[P_S]$ and $[P_L]$ are the activities of the poly-ions in the resin and the liquid phase respectively, $[Na_S^+]$ and $[Na_L^+]$ the activity of the sodium ions in resin and liquid phase, application of the law of mass action results in:

$$\frac{[P_S]\,[Na_L^+]^z}{[P_L]\,[Na_S^+]^z} = K \tag{2b}$$

The adsorption coefficient C is proportional to $[P_S]/[P_L]$ $(= K[Na_S^+]^z/[Na_L^+]^z)$ and therefore as a rough approximation:

$$C = K_1 \times \left\{\frac{\text{Na}^+ \text{ adsorbed by resin}}{[Na_L^+]}\right\}^z \tag{2c}$$

Equation (2c) indicates that the ion-exchange adsorption of poly-ions on a cation exchange resin is more sensitive to slight changes in cation concentration than that of monovalent ions. It is to be expected that the decrease of the adsorption coefficient with rising cation concentration will be steeper. The findings of BOARDMAN AND PARTRIDGE concerning the adsorption of cytochrome *c* (iso-electric point 10.1) at pH 7 were again in good agreement with their expectation[8].

However, when the adsorption was plotted as a function of pH it was found that at higher pH values the adsorption decreased steeply with increasing pH, while on lowering the pH it did not decrease but became very strong below pH 7, as can be seen from Fig. 8 (taken from BOARDMAN AND PARTRIDGE).

It was observed that the rapid rise of the adsorption coefficient below pH 7 coincided with a diminution of the amount of sodium ions taken up by the resin. Therefore it was believed that in this pH region adsorption is no longer caused by pure electrostatic forces, but chiefly by short-range forces such as those giving rise to hydrogen linkages, mainly between undissociated carboxyl groups of the resin and peptide bonds or other polar groups of the protein. The positive charge of the protein, which above pH 7 is probably the main cause of adsorption, rapidly becomes unimportant below that pH.

As for the haemoglobins (iso-electric points around pH 7 in 0.1 *M* phosphate buffer, and about 5 in 0.1 *M* citrate buffer), it can be expected that adsorption will always be due to secondary forces. In the pH region where these short-range forces become unimportant, there is no electrostatic attraction, because above pH 7 haemoglobins carry a negative net charge. Therefore, chromatography of haemo-

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globins must be carried out in a pH region somewhere between 5 and 7. Differences in adsorption coefficients will arise partly from differences in short-range forces (due to differences in the spatial structures) and partly from differences between electrostatic attraction or repulsion forces (due to differences in net charge at a certain pH).

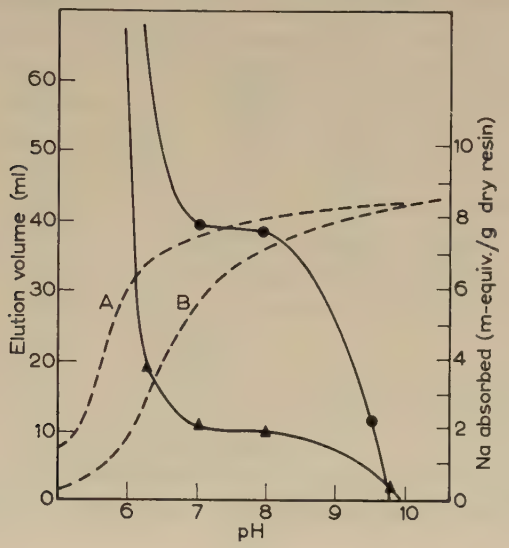

Fig. 8. Effect of pH on the elution of cytochrome *c* by sodium phosphate buffers. 6.0 mg samples were chromatographed on columns of IRC-50, 16.0 × 0.9 cm. Temperature 25°. Na^+ concentration: ● 0.25 g.ions/l; ▲ 0.34 g.ions/l. The broken lines show the amount of Na^+ taken up by the resin: A, from 1.0 *M* NaCl; B, from 0.1 *M* NaCl (BOARDMAN AND PARTRIDGE[8]).

Optimal conditions for a good separation in a given mixture have to be found empirically.

In haemoglobin chromatography on Amberlite IRC-50 the following procedure is generally followed.

Pre-treatment of the resin. The best way to make the commercial Amberlite IRC-50 XE-64 or 97 (passing a 150-mesh/in. sieve) ready for use has been described in detail by HIRS, MOORE AND STEIN[19]:

In order to remove the very fine particles, which would block the column, the following procedure is repeated several times: 1500 g of resin are stirred mechanically for 20 min with 3½ l of distilled water and then allowed to settle for 20 min after which the cloudy supernatant is decanted.

After this procedure the resin is stirred for 3 h in 3 l of acetone; fine air bubbles evolve and a yellow product is extracted. The ion-exchange resin is then cycled once or twice through the sodium form and finally stored, either in the acid form in aqueous suspension or in a concentrated buffer solution, or suspended in a NaOH solution. Regeneration of the resin is best carried out by heating for 6 h in 4 *N* HCl, washing with water, heating for 6 h in 4 *N* NaOH solution and again washing with water.

Before use, the purified resin can be roughly equilibrated with the buffer used

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in the experiment, by repeatedly suspending it in a large excess of buffer. Alternatively, the acid form of the resin can be used without pre-equilibration; saturation with the buffer is then performed on the column.

Preparation of the columns. The columns are prepared at the temperature at which the chromatographic experiment will be carried out. A suspension of the resin

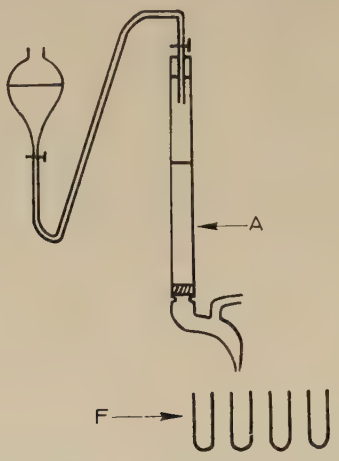

Fig. 9. Assembly for elution chromatography. A = adsorbent. F = fraction collector.

in 2 to 3 volumes of buffer solution or distilled water is poured, in small portions at a time, into a vertical glass tube fitted with a sintered glass disk at the lower end. After the first portion of the resin has settled, more of the slurry is poured into the tube, until after settling the desired column height has been reached. A separatory funnel containing the developing buffer saturated with carbon monoxide is connected to the tube (Fig. 9) and buffer is passed through the column at a high flow rate until the pH of the effluent is equal to that of the buffer entering the column. Then, by lowering the separatory funnel, the flow rate is adjusted to the value that will be used in the experiment (½–¾ ml/h per cm^2) and maintained for several hours, a check also being kept on the pH during that time. Columns prepared in this way, can be stored for some weeks, but only in the cold and if care is taken that there is always some buffer solution above the resin surface.

Chromatographic procedure. At the beginning of a chromatographic experiment the buffer solution standing above the resin surface is allowed to drain into the column. As soon as the surface is "dry", the sample to be chromatographed is added very carefully, drop by drop, to avoid disturbance of the resin surface. The sample consists of a 1–4% solution of HbCO in water, the volume employed for a 1 × 10 cm column being ½–1 ml. The HbCO solution is allowed to drain into the column by gravity, then about 1 ml of developing buffer is dropped carefully on the resin surface, after which a small plug of cotton wool is placed on the top of the column (to protect the resin surface from being disturbed by the next manipulations); then the glass tube is rapidly filled with buffer and finally connected with the separatory funnel.

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The haemoglobin concentration in the effluent is estimated by diluting the ¼ or ½ ml fractions to 4 ml with distilled water and measuring the optical density at 570 mμ, or, for low concentrations, at 418 mμ (Soret line) in a spectrophotometer.

All results reported by different investigators have been obtained with this, or a very similar, procedure.

The results obtained by BOARDMAN AND PARTRIDGE[8] with mixtures of sheep foetal HbCO and bovine HbCO are very instructive. Fig. 10 shows elution curves of experiments performed at 25° and 2°. In both diagrams a third peak can be seen besides the two peaks corresponding to the two components of the mixture (peak A being sheep foetal HbCO and peak B bovine HbCO). This third peak was obtained by eluting the brownish material that was left behind on the column by both components. From the absorption spectrum of the material in the third peak it was concluded that it consisted of methaemoglobin. It was found that formation of methaemoglobin could be suppressed by working at low temperature with freshly prepared HbCO solutions.

In experiments with human haemoglobins, using mixtures of human adult and foetal HbCO, we[30, 62] obtained diagrams such as that shown in Fig. 11. It can be seen that the two haemoglobin types separated very well; Hb-A is, however, eluted in much lower concentration than Hb-F. In the same buffer solution good separations could also be obtained of mixtures of HbCO-A and HbCO-S (sickle-cell Hb). Hb-S

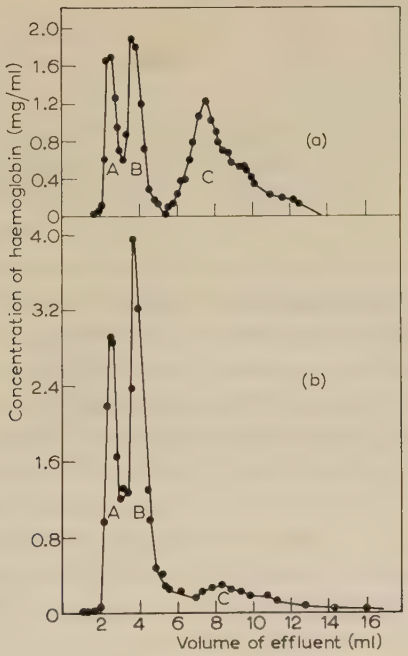

Fig. 10. Chromatograms of a mixture of sheep foetal HbCO and bovine HbCO on 0.9 × 5.0 cm Amberlite IRC-50 columns. Buffer: sodium citrate pH 5.81, Na^+ conc. 0.34 g.ions/l; after 1.2 ml changed to sodium citrate pH 6.5, Na^+ conc. 3.2 g.ions/l. (a) Temperature 25°; (b) Temperature 2° (BOARDMAN AND PARTRIDGE[8]).

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proved to be more strongly adsorbed than Hb-A; it could be eluted in a concentration comparable with that of Hb-A when a pH 6.5 citrate buffer, containing 0.25 g.ion Na^+/l, was used. The fourth haemoglobin type investigated was Hb-C, which showed the strongest adsorption that has been observed until now; it could be eluted with a pH 6.5 citrate buffer, 0.30 g.ion Na^+/l. The yields of HbCO could be improved from about 85% at 10° to almost 100% at 0°. We used the chromatographic procedure to

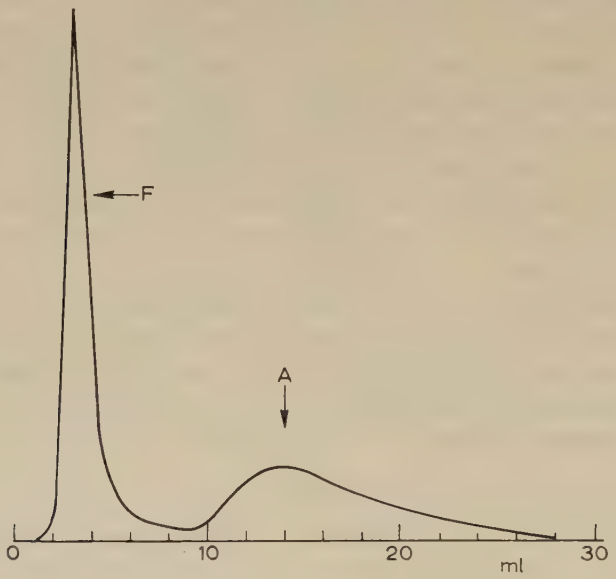

Fig. 11. Chromatogram of a mixture of human adult and foetal HbCO on a 0.9 × 7 cm Amberlite IRC-50 column. Temperature 10°; rate of flow 1.2 ml/h. Total amount of haemoglobin approximately 5 mg. Buffer: Solution of 10.0 g citric acid·1 aq., brought to pH 6.5 by addition of NaOH, diluted to 1 l. Na^+ concentration made up to 0.20 g.ions/l by addition of solid NaCl.

isolate Hb-F from the blood of a patient suffering from Cooley's anaemia, in order to estimate the amino-acid composition of this haemoglobin[31].

Besides being used for the purification of different haemoglobin types before further characterisation, chromatographic procedures may be of importance in the identification of abnormal haemoglobins in clinical serial analyses. The procedure described in the foregoing part is, however, still too complicated and time-consuming to be suitable for routine analysis. Therefore we simplified the method in the following way[25, 30, 62]:

The cylindrical glass tubes are replaced by flat Lucite (Perspex) cuvettes (20 × 3 × 0.5 cm inside dimensions) placed in a rack (Fig. 12) in which they can easily be moved upwards or downwards. The cuvettes are connected with the buffer vessel by means of siphons.

The chromatographic procedure is as follows:

Amberlite columns of 15 cm height are prepared as described above (p. 146). The upper few mm of resin are equilibrated with 25 ml of the developing buffer

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(a 20-fold dilution of a sodium citrate–citric acid solution, pH 6, containing 1 mole of trisodium citrate per l). The resin is further equilibrated with the buffer during the development of the chromatogram. As a result of the strong buffering properties of the resin (it contains about 10 mequiv. H^+ ions per g dry weight, that is about 2 mequiv. H^+ per cm column height), a pH and Na^+ concentration gradient develop on the column during chromatography. At first this gradient is very steep, but it becomes flatter with time. After passage of 250 ml of the developing buffer, however, the pH difference between the top and the bottom of the cuvette is still 0.5 pH unit[63].

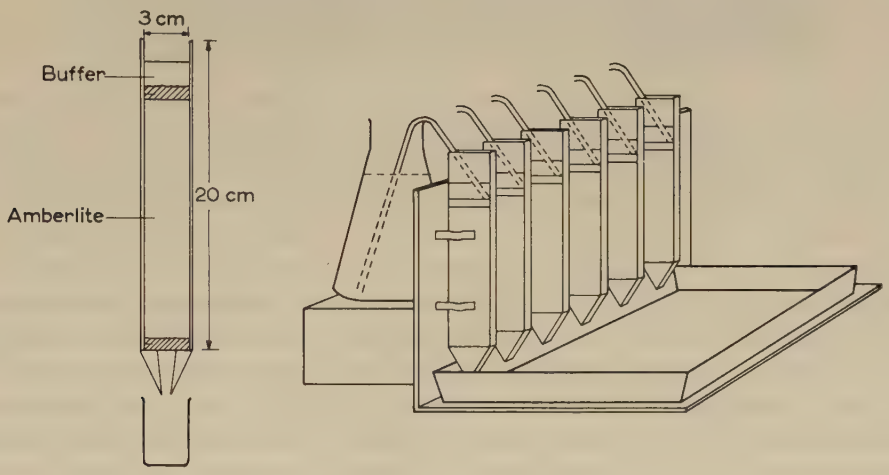

Fig. 12. Technical equipment for chromatography in flat cuvettes.

After 25 ml of buffer have passed through the column, 2–4 ml of an aqueous solution of 10–15 mg HbCO are carefully applied on the surface of the resin and allowed to drain into the column; then about 5 ml of citrate buffer are carefully applied. When there is still a layer of buffer solution above the resin surface, this surface is protected by placing a piece of cotton wool on it. Finally the cuvette is connected with the buffer vessel by means of a siphon and the rate of flow is adjusted to about 10–15 ml/h. It is advisable to place the whole assembly in a cold room or refrigerator at 0–4°, to avoid methaemoglobin formation (see Fig. 14).

When 200 ml of buffer solution have passed through, a good separation of an artificial mixture of Hb-A and Hb-F or Hb-A and Hb-S is in general obtained: two horizontal well separated zones become visible. In Fig. 13, separations carried out with some artificial and naturally occurring mixtures are shown. It appeared that when cuvettes were used under the conditions described above, very good separations of haemoglobins F, A, S and C could be obtained; the relative migration velocities proved to be fairly constant. If the rate of displacement for Hb-A is taken as 1.0, the rate for Hb-F is 1.3; for Hb-S 0.7; for Hb-C 0.3.

The separation of Hb-E from Hb-A is attended by more difficulties; a good separation could only be realized when a low rate of flow (5 ml/h) of the developing

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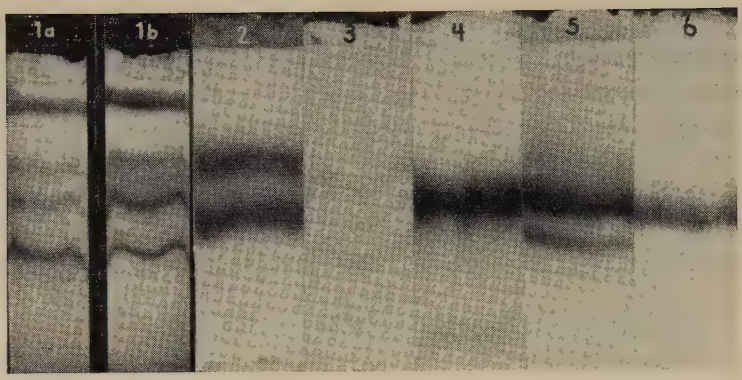

Fig. 13. Cuvette chromatograms of various mixtures of human haemoglobins. 1a: Artificial mixture of haemoglobins F, A, S and C (named in order of decreasing rate of displacement). 1b: Artificial mixture of haemoglobins F, A, E, S and C (also named in order of decreasing rate of displacement). 2: D-trait haemoglobin. 3: Hb-E thalassaemia disease. 4: Haemoglobin from Hb-H trait. 5: Haemoglobin from Hb-I trait. 6: Haemoglobin from Hb-J trait (no separation).

buffer was maintained. The Hb-E then becomes visible as a somewhat slower migrating zone, compared with that of Hb-A (Fig. 13), but the two zones are scarcely separated. Of the other haemoglobins investigated, Hb-D cannot be distinguished from Hb-S, Hb-H migrates more rapidly than Hb-F, Hb-I has a velocity between those of Hb-A and Hb-F and can hardly be separated from Hb-A; Hb-J cannot be distinguished from Hb-A by the cuvette method; Hb-L has a rate of displacement between those of Hb-S and Hb-C. The role of the cuvette method in the qualitative analysis of unknown haemoglobin mixtures will be discussed later (p. 165).

Semi-quantitative information can be obtained in various ways:

(1) By determining the optical density by scanning diapositives of the cuvettes, as in the direct scanning of paper electrophoresis strips[17]. Some scanning diagrams are given in Fig. 14. It can be seen that the results are far better at 0° than at 20°. Methaemoglobin formation in particular proves to be largely suppressed at low temperatures.

(2) By means of a more rapid procedure in which cuvettes with a removable front plate are employed. After development of the chromatogram, the front plate of the cuvette is detached, the separated zones are cut out and eluted from the resin into separate small chromatographic glass tubes with a more concentrated citrate buffer (a 3-fold dilution of the stock solution described on p. 149). The concentration of haemoglobin in the eluates is estimated photometrically. The method is of course also suitable for small scale preparative work.

By both estimation methods, relative amounts of haemoglobins in mixtures as low as 10% can be measured with reasonable accuracy. Zones containing ½ mg Hb (in general constituting 5% of the total amount of Hb) can be detected by visual inspection but cannot be easily measured.

In many cases, when the flow rate of the developing buffer is low, a second zone besides the main fraction becomes visible in a chromatogram of normal adult Hb

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and also of cord blood Hb; this second zone migrates with a somewhat higher velocity. In Fig. 15, the secondary fractions of Hb-A and Hb-F (designated as Hb-a and Hb-f respectively) can be seen. The occurrence of these zones can lead to wrong interpretations of chromatograms; Hb-a can be mistaken in this way for Hb-F. Any uncertainty can, however, always be resolved by adding a small amount of cord blood haemoglobin to the mixture in a second run, because this gives rise to a second small zone in front of the zone originally taken for foetal Hb.

We isolated the secondary fraction from normal adult Hb, by applying 1 ml 10% HbCO or HbO_2 to a 2½ × 15 cm column of Amberlite IRC-50 XE-64 equilibrated with citrate buffer pH 6.5 (0.20 g.ion Na^+/l). In normal adults the relative concentration of the minor fraction proved to be about 10% (for 4 samples the values found were 9.0, 11.7, 11.1 and 10.9% respectively). In some patients with anaemia a higher percentage (15–20%) was found. On rechromatography the Hb-a fraction retained its rapid rate of displacement, as compared with the main fraction; it showed no deviations from the main fraction as far as absorption of visible and U.V. light or denaturation velocity in alkaline solution were concerned. In paper electrophoresis it migrated somewhat more rapidly to the anode than the main fraction; in this respect and also in chromatography, it resembles the abnormal Hb-I very closely. Whether or not Hb-a and Hb-I are identical cannot be said as yet.

Somewhat different results were obtained by MORRISON AND COOK[55], on chromatographing 50 mg of HbO_2 on 0.9 × 50 cm Amberlite IRC-50 columns at 4°. The haemoglobin was eluted with phosphate–citrate buffer (pH 6.3) of constantly increasing ionic strength from 0.03 *M* to a maximum of 0.2 *M*; the flow rate was

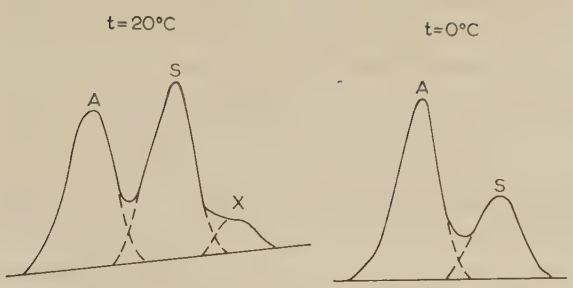

Fig. 14. Optical density curves obtained from cuvettes, in which mixtures of Hb-A and Hb-S have been chromatographed at 20° and 0° resp. Note the peak in the left hand diagram marked with an x, which is caused by a brownish zone, probably methaemoglobin.

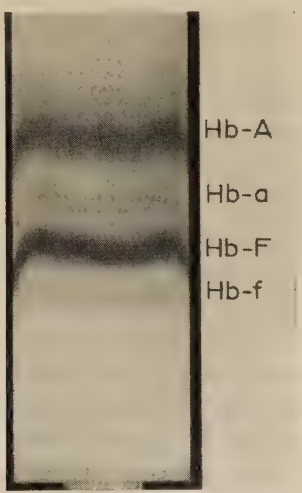

Fig. 15. Main and secondary fractions in a mixture of normal adult and fetal haemoglobin.

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approximately 10 ml/h. With haemoglobin from normal adults, MORRISON AND COOK obtained 3 different fractions, the first representing about 10% of the total (possibly identical with the Hb-a component found by us[62]), the second and third constituting 84 and 6% of the total respectively. Each of the fractions gave an absorption spectrum typical for oxyhaemoglobin. The investigators do not report whether the different components were rechromatographed.

Operating under different conditions, ALLEN, SCHROEDER AND BALOG[2] investigated crystallized as well as uncrystallized normal human adult red cell haemolysates. Besides the main fraction (comprising about 90% of the total haemoglobin), they obtained various subfractions, which all exhibited a higher rate of displacement on the column than the major fraction. Under the conditions of the experiment HbO_2 and ferrihaemoglobin cyanide showed identical chromatographic behaviour. ALLEN *et al.*

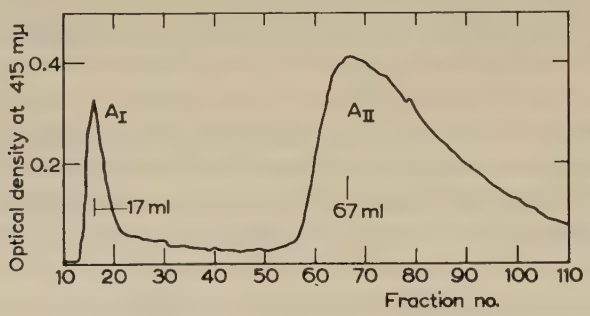

Fig. 16. Chromatogram of normal adult oxyhaemoglobin on a 1 × 35 cm column of IRC-50 Developer: phosphate buffer, containing 3.45 g $NaH_2PO_4 \cdot H_2O$, 2.66 g Na_2HPO_4 and 0.65 g KCN/l. The pH at 25° is 7.18 ± 0.02; the solution contains 0.0625 g.ions Na^+/l and 0.01 g.ions K^+/l. Temperature, 5–6°; rate of flow, 5 ml/h; fraction volume, 1 ml. The fractions were diluted to 3 ml before spectrophotometric reading. (ALLEN, SCHROEDER AND BALOG[2]).

employed Amberlite IRC-50 of very uniform particle size: the resin was sifted wet in the hydrogen form and the fraction 200–250 mesh was used for preparing the columns (dimensions 1 × 35 cm for analytical work). The columns were employed for several successive experiments. For reasons of stability the elution buffers chosen were of higher pH (7.0–7.2) than those used by BOARDMAN AND PARTRIDGE (5.8–6.5), by us (6.0–6.5) or by MORRISON AND COOK (6.3). Consequently, the sodium ion concentrations of the buffers had to be lower (0.05–0.075 g.ion Na^+/l) than in earlier investigations, in order that the haemoglobin should be adsorbed. Potassium cyanide was added to the buffer to convert any methaemoglobin formed during the experiment into ferrihaemoglobin cyanide, which does not differ from HbO_2 as regards chromatographic behaviour and therefore does not produce additional zones on the column. The haemoglobin sample (containing 30–60 mg Hb for chromatography on 1 × 35 cm columns) was dialysed against the developing buffer before the chromatographic run, which was carried out at 5–6° with a flow rate of 5 ml/h. For rechromatography, only the most concentrated effluent that contained a zone was used and not the entire effluent.

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In Fig. 16 a chromatogram of a fresh haemolysate of adult red cells is presented. Under the conditions of the experiment part of the haemoglobin (about 10%) is not adsorbed by the resin and can be easily and completely separated from the main fraction. No third component is visible. When the non-adsorbed fraction (designated A_I), was rechromatographed under somewhat different conditions (lower pH and ionic strength), 3 fractions (A_{Ia}, A_{Ib}, and A_{Ic}) were obtained besides some non-haem protein. Spectral studies revealed that during rechromatography some methaemoglobin cyanide had been formed.

Chromatography of cord blood oxyhaemoglobin with an elution buffer of pH 7.22 (0.075 g.ion Na^+/l) resulted, as in the first experiment with adult Hb, in a separation into two fractions: one was not adsorbed by the column and turned out to be foetal Hb, the other showed a rate of displacement identical with that of the main component in the experiment with adult Hb. Rechromatography of the foetal fraction with the same buffer that was used for the rechromatography of zone A_I, resulted in separation into two fractions: the first fraction eluted (designated F_I) constituted about 20% of the foetal Hb. Whether any subfraction of zone A_I is identical with a subfraction of zone F has not been established.

Table 2 gives some results of phenylalanine, leucine and isoleucine determinations in the different haemoglobin fractions prepared by ALLEN *et al.*

TABLE 2

AMINO ACID ANALYSES OF SOME HAEMOGLOBIN PREPARATIONS AND FRACTIONS (ALLEN *et al.*[2])

Preparation	*Ratios*	
	leu/phe	*ileu/leu*
Haemoglobin uncrystallized	1.9	0.014
Oxyhaemoglobin, crystallized	1.9	0.013
Carbonmonoxyhaemoglobin, crystallized	1.9	0.015
Oxyhaemoglobin, crystallized twice fractionally	1.9	0.017
Main peak of electrophorised haemoglobin	1.9	0.000
Trailing edge of electrophorised haemoglobin (including non-haem protein)	1.9	0.11
Zone A_{II}	1.9	0
Electrophorized zone A_I	1.8	0.037
Non-haem trailing edge of electrophorised zone A_I	1.4	0.34
Non-haem protein obtained by chromatography	1.6	0.46
Zone F_{II}	1.8	0.10
Zone A_{Ic}	2.0	0.044
Globin	1.8	0.013

ALLEN, SCHROEDER AND BALOG draw the following conclusions from their experiments:

(1) Both crystallized and uncrystallized adult haemoglobin contain, in addition to the main component, at least 3 haem proteins and 1 non-haem protein. The various components are not artifacts formed during preparation or chromatography; they differ in their isoleucine content.

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(2) None of the haem proteins correspond to the slowly moving subfraction found by MORRISON AND COOK.

(3) None of the haem proteins could be definitely identified with foetal haemoglobin.

(4) Cord blood haemoglobin also contains minor components.

The authors make the following suggestion: "If the (chromatographic) methods were applied to the haemoglobin mixtures that are known to occur in certain pathological conditions, it probably would be possible to determine whether entirely different components are present or whether components normally present in minor amount have simply been increased proportionately".

Apart from the conclusions concerning the heterogeneity of adult red cell haemolysates, the investigations of ALLEN *et al.* are very important because they show that good preparative separations can be obtained in complex mixtures by chromatographing in several runs; this had already been suggested by BOARDMAN AND PARTRIDGE[8]. In the first run the mixture is divided into an adsorbed and an unadsorbed portion; in the following run or runs the non-adsorbed fraction is fractionated under slightly more acidic conditions and with a lower ionic strength. With this procedure there is less risk of denaturation occurring than with those in which all the components of a complex mixture are adsorbed and then desorbed one by one.

Adsorption on carboxymethyl cellulose

Ion-exchange adsorbents derived from cellulose which were introduced by PETERSON AND SOBER, are becoming more and more important in the chromatography of proteins, nucleoproteins and nucleic acids. PETERSON AND SOBER[60] prepared different types of cation and anion exchangers from cellulose powder, for instance by reaction with monochloracetic acid (product: carboxymethyl cellulose), with diethyl-2-chloroethyl-amine (product: diethylaminoethyl cellulose). The last-mentioned product, an anion exchanger, was first used by PETERSON *et al.*[78] in the chromatographic separation of serum proteins. Preliminary experiments gave promising results.

Recently, HUISMAN *et al.* employed the cation exchanger carboxymethyl cellulose in very successful experiments with different types of human haemoglobin[24]. The results of these experiments will be discussed in this section.

There are very marked differences between the cation exchangers Amberlite IRC-50 (discussed in the former section) and the carboxymethyl cellulose adsorbents.

(1) To make sure that the carboxymethyl cellulose (CMC) will not be water-soluble in the sodium form, such reaction conditions are chosen for the preparation of the adsorbent that only few carboxyl groups are introduced (according to PETERSON AND SOBER CMC contains about 0.5–1.0 COOH/g dry weight; Amberlite IRC-50 contains about 10 mequiv. COOH/g dry weight).

Consequently, CMC adsorbent has a very low buffer capacity, so that it is suitable for gradient elution experiments; only the molarities and the pH of the elution buffers are of importance. A certain disadvantage of the low buffer capacity

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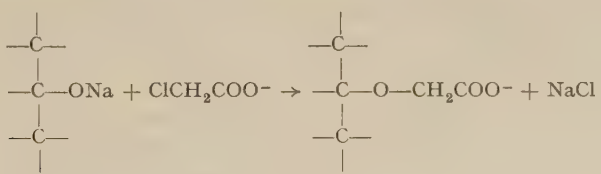
is the necessity to dialyse the protein sample very carefully against the first elution buffer before starting the chromatographic experiment. In chromatography on Amberlite IRC-50 this is not necessary. Column artifacts are easily formed as a result of the low buffer capacity of the cellulose adsorbent: when a protein solution is applied to a CMC column under such conditions that the protein is just adsorbed but a small increase in ionic strength of the buffer solution will cause desorption, it is often observed[23, 63] that the first portion of the protein sample is adsorbed at the top of the column; this adsorption, however, releases so many cations from that part of the column that the rest of the column is in equilibrium with a higher cation concentration, which results in non-adsorption of the last part of the protein sample applied to the column. In this way pseudo-heterogeneity can be observed in protein samples that are in fact homogeneous. The occurrence of such artifacts can be avoided by adsorbing under less critical conditions.

(2) Carboxymethyl cellulose can adsorb reversibly its own dry weight of protein, whereas Amberlite IRC-50 because of its high percentage of cross-linkages can only adsorb protein on the particle surface, that is a few milligrams of protein per gram dry weight. Adsorption on CMC is reversible, probably because the number of carboxyl groups per gram adsorbent is low. There are enough carboxyl groups present for adsorption to occur, but not so many that strong multipoint attachment will result in irreversible deformation. This is another feature that makes CMC more suitable for gradient elution chromatography than other adsorbents known so far. At the beginning of an experiment the various components of a complex mixture can be run on a column at such a rate that the R_F values are much lower than 0.5, without the risk of denaturation occurring caused by too strong adsorption; the components can then be desorbed one after another by slightly increasing the buffer concentration.

Apart from the differences mentioned here, the considerations of BOARDMAN AND PARTRIDGE concerning protein adsorption by Amberlite IRC-50 are qualitatively also valid for CMC (p. 143).

CMC is prepared by means of the reaction of Williamson:



Procedure. According to PETERSON AND SOBER[60], a solution of 90 g NaOH in 200 ml water is stirred into 60 g of Whatman cellulose powder that has passed a 325 mesh sieve. The paste obtained is cooled in an ice bath for half an hour under occasional stirring*. A solution of 30 g monochloroacetic acid in 40 ml water is added slowly under mechanical stirring. While stirring is continued the beaker containing the reaction mixture is immersed for 20 min in a 70° oil or water bath, after which

* It is our experience that if an additional 100 ml of water is added the mass obtained can more easily be stirred.

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it is transferred to an ice bath. After cooling, 500 ml of 10% acetic acid is added slowly, also under mechanical stirring. The yellow reaction mixture, which is still alkaline, is then poured into 1 l of distilled water and allowed to settle for at least 3 h. The cloudy yellow supernatant is removed, the sediment is shaken or stirred with a fresh portion of distilled water and again allowed to settle. This washing procedure is continued until the suspension is no longer yellow. The acid form of the adsorbent is obtained by adding 10 ml glacial acetic acid to the washed suspension, filtering on a coarse sintered glass or Buchner funnel, washing first with water and then with ethanol and drying at room temperature *in vacuo*.

SOBER AND PETERSON report a yield of 45 g of white powder, containing 0.7 mequiv. acidic groups per g*.

Columns of CMC are prepared in the same way as described for Amberlite IRC-50 (p. 146). They are also equilibrated with buffer by passing buffer through the column until the pH of the inflowing buffer and the effluent are exactly equal.

HUISMAN *et al.*[24] employed glass tubes (55 × 0.9 cm) filled up to a height of 52 cm with CMC. pH-gradient elution chromatography was performed as follows:

The column was first equilibrated with a 0.01 *M* phosphate buffer, pH 6.8 (sometimes pH 6.5, 6.3 or 6.0). The phosphate buffer was always a mixture of two solutions, containing 1.78 g $Na_2HPO_4 \cdot 2$ aq. per l, and 1.56 g NaH_2PO_4 per l, respectively. After the introduction of a solution of 100–150 mg of HbCO in a volume of 5 ml (the sample was first dialysed for 24 h against the starting buffer at 4°), the glass tube was filled to the top with the starting buffer, then connected with a mixing chamber (50 ml) which also contained the starting buffer. The mixing chamber was connected with a supply bottle containing 0.01 *M* phosphate buffer pH 8.0 (prepared in the same way as described for the starting buffer). A flow rate of 4–6 ml/h was maintained throughout each experiment; the temperature was kept below 10°.

By this procedure several excellent separations were obtained in various mixtures. The recovery was always higher than 97%.

Normal adult haemoglobin (preparation see p. 131). An elution diagram of normal adult red cell haemolysate is presented in Fig. 17a. After 25 ml have run through, the pH curve shows a maximum, possibly as a result of sodium ions being displaced from the adsorbent by protein (p. 155). HUISMAN *et al.*[24] suggest the possibility that the first component (V), generally constituting ½–1% of the total amount of haemoglobin, does not really exist, and that the corresponding peak is caused by the initial rise of the pH. As soon as the pH of the eluent is 7.40 ± 0.01, a fraction (A_1) (10% of the total amount of Hb) is eluted. This fraction is probably identical with the rapidly migrating subfraction obtained in Amberlite IRC-50 chromatography (p. 466). The main fraction A_0 is eluted at pH 7.49 ± 0.01, followed at pH 7.69 ± 0.01 by the last (A_2-fraction) peak, containing 0.5–2.0% of the total Hb; this last fraction is not seen in Amberlite chromatography. Paper electrophoresis on Whatman No. 3 MM

* We[63] obtained CMC batches containing respectively 0.46, 0.48 and 0.45 mequiv. acidic groups per g dry weight; possibly these lower values resulted from the dilution of the reaction mixture at the beginning of the procedure (see footnote*).

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paper at pH 8.6 (for description see p. 135) showed that fractions A_1 and A_2 behaved electrophoretically as Hb-J and Hb-E, respectively; A_1 migrated somewhat faster than Hb-A and A_2 much slower than this substance. The authors suggest that these subfractions may be identical with the electrophoretical subfractions obtained by KUNKEL AND WALLENIUS (p. 139).

A chromatogram of a Hb sample of a patient with Hb-J trait reveals that the abnormal haemoglobin is eluted at almost the same pH (7.39) as Hb-A_1 (7.40). This suggests that the so-called abnormal Hb-J might be identical with a minor fraction (A_1) of normal adult haemoglobin. However, it should be borne in mind that by chromatography on Amberlite IRC-50 cuvettes the faster moving subfraction could be separated from the main fraction, whereas Hb-J did not separate from Hb-A. Thus the possibility exists that either the faster moving Amberlite-fraction Hb-a is not identical with the CMC-fraction A_1, or that the CMC-fraction Hb-A_1 is not identical with Hb-J. This can perhaps be checked by estimation of the amino acid composition of the different haemoglobin fractions.

Another possible identity is that of fraction A_2 and the abnormal haemoglobin E. Electrophoresis revealed no difference, and chromatography of a haemoglobin sample of Hb-E trait blood on CMC showed that Hb-E was eluted at the same pH (7.68) as Hb-A_2 (7.69) (Fig. 17c). It is very likely that the CMC-fraction Hb-A_2 is identical with the Hb-A_2 fraction obtained electrophoretically, for the following reasons: their electrophoretic mobilities are the same, the percentages present in normal adults are of the same order, and these percentages are raised to almost the same extent in patients with Cooley trait.

Cord blood haemoglobin. Fig. 18b is an elution diagram of cord blood haemoglobin

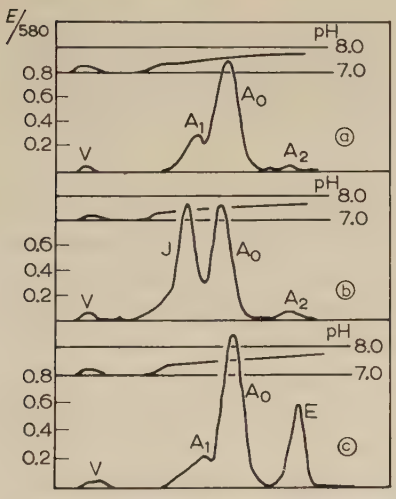

Fig. 17. pH-gradient elution diagrams of some haemoglobin samples chromatographed on CMC columns. (a) Normal adult HbCO; (b) HbCO from Hb-J trait; (c) HbCO from Hb-E trait. (HUISMAN, MARTIS AND DOZY[24]).

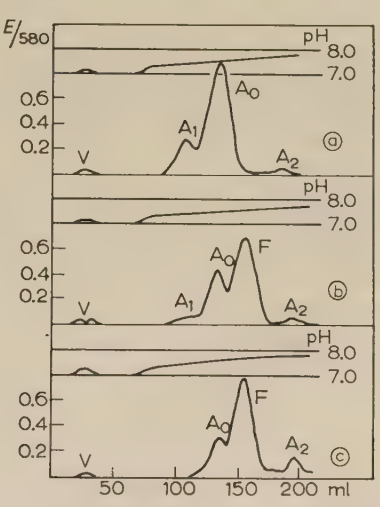

Fig. 18. Behaviour of foetal haemgolobin in pH-gradient CMC chromatography. (a) Normal adult HbCO; (b) HbCO from full term infant; (c) HbCO from three month old premature infant. (HUISMAN, MARTIS AND DOZY[24]).

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of a normal full term infant; besides *one* well-separated Hb-F peak, all adult components A_1, A_0 and A_2 are present. These results differ in some respects from those of ALLEN *et al.* obtained with Amberlite IRC-50 chromatography. In the latter case foetal components were recognized and only one adult component. In special cases fraction A_1 is not observed in CMC chromatography, while fraction A_2 is then increased several fold (Fig. 18c).

It is further remarkable that Hb-F has a lower rate of displacement than Hb-A_0 on CMC columns. In Amberlite chromatography it moves faster than Hb-A.

Behaviour of some abnormal haemoglobins. Besides the investigation of Hb-J and Hb-E elution experiments were also carried out with mixtures containing the haemoglobins S, C, H, L, as well as a newly detected haemoglobin, designated Buginese X.

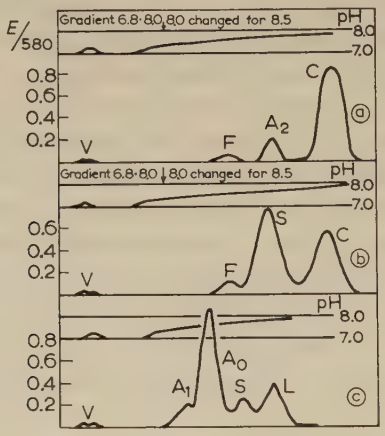

Fig. 19. pH-gradient elution diagrams of various mixtures containing abnormal haemoglobins, chromatographed on CMC columns. (a) Homozygous Hb-C; (b) Haemoglobin S–haemoglobin C disease; (c) Heterozygous Hb-L, after addition of some Hb-S. (HUISMAN, MARTIS AND DOZY[24]).

Elution diagrams are presented in Fig. 19. It can be seen that each abnormal haemoglobin can be identified by the distinct pH at which it is eluted. It is further remarkable that CMC elution diagrams reveal more details than other techniques employed so far. Data collected by HUISMAN and co-workers when studying different normal and abnormal red cell haemolysates are listed in Tables 3 and 4.

From Table 3 it can be seen that except for Hb-L, all haemoglobin types investigated are eluted in the same order as their migration velocities towards the anode in electrophoresis at pH 8.6, and also in the same order as their iso-electric points (as far as they are known). The elution pH is always 0.4–0.6 pH units higher than the iso-electric point. The results of the pH-gradient elution experiments of HUISMAN *et al.* suggest that the separations are mainly caused by differences in electrostatic attraction at various pH values. As soon as a haemoglobin type has passed its iso-electric point, electrostatic repulsion forces very rapidly become more

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TABLE 3

BEHAVIOUR OF DIFFERENT HAEMOGLOBIN TYPES IN CMC CHROMATOGRAPHY AND ELECTROPHORESIS AT pH 8.6

	Hb type									
	H	*J*	A_1	A_0	*F*	*L*	*S*	*E*	A_2	*C*
Elution pH in CMC chromatography	6.7 — 6.9	7.39 ± 0.01	7.40 ± 0.01	7.49 ± 0.01	7.58 ± 0.01	7.71	7.64 ± 0.02	7.68 0.01	7.69 0.01	7.82
Migration velocity to the anode at pH 8.6	H >	J >		A_0 >	F >	L >	S >	E =	A_2 >	C
Iso-electric point	6			6.87	6.98		7.09	7.20		7.30

TABLE 4

THE PERCENTAGE AMOUNT OF DIFFERENT HAEMOGLOBIN COMPONENTS IN VARIOUS RED CELL HAEMOLYSATES

Type of blood	*Number of cases*	*V*	A_1	A_0	A_2	*F*	*Abnormal component*
Normal adult	7	1.5 (1–2)	10.5 (8–15.5)	87 (84–90[5])	1 (0.5–2)	—	—
Cord blood	3	1 (0–1[5])	2 (0–2[5])	22 (19[5]–25[5])	0.7 (0[5]–1)	75 (69–79[5])	—
Cord blood (premature)	1	2	0	11	6	81	—
Cooley's trait	2	1	6.5	86.5	6	—	—
		1.5	12	81.5	5	—	—
Cooley's anaemia	2	1	4	17	1	77	—
		1	12.5	55.5	0.5	30.5	—
Sickle-cell anaemia (after blood transf.)	1	0.5	4.5	21	5.5	7.5	Hb-S: 61%
Hom. Hb-C disease	1	0.5	—	—	8.5	3.5	Hb-C: 87.5%
Hb-S/Hb-C disease	1	0.5	—	—	?	5.5	Hb-S: 49.5% Hb-C: 44.5%
Hb-E trait	4	1 (0.5–2[5])	10 (8[5]–11)	65.5 (62–71[5])	—	—	Hb-E: 22[5]% (16–28.5)
Hb E thalassaemia	3	1 (0–1[5])	3[5] (0–6[5])	18 (10–30)	—	39 (30–52[5])	Hb-E: 38% (32[5]–48)
Hb H disease	1	—	11	73	1	—	Hb-H: 15%
Hb J trait	1	1	?	45.5	2	—	Hb-J: 51.5%
Hb L trait	1	0.5	9.5	65	?	—	Hb-L: 25%

important than the short range forces, which hold the haemoglobin, now negatively charged, to the also negatively charged adsorbent.

It would be interesting to perform chromatographic experiments with haemoglobin on CMC columns, working with a buffer concentration gradient at a fixed pH (for instance pH 6 or 6.5 as in the chromatography on Amberlite IRC-50 columns).

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At these low pH values, secondary range forces will perhaps become relatively more important, and differences in structure rather than differences in electric charge will be reflected in the elution diagrams.

On comparing pH gradient elution on CMC columns with chromatography on Amberlite columns, we observed that, in general, elution curves of various mixtures of haemoglobins adsorbed by CMC show more detail than chromatography of the same mixtures on Amberlite at a fixed pH. Moreover, every component is eluted at a highly reproducible pH value; this value is a characteristic of a certain haemoglobin component. However, it should be borne in mind that although the values were reproducible for the adsorbent prepared by HUISMAN *et al.*, it is not yet known whether deviating elution pH values will be found on columns prepared from other batches of carboxymethyl cellulose. On the other hand the characteristics of the haemoglobin types in Amberlite chromatography (elution volumes and rates of displacement at fixed pH) are much less reproducible, for they are highly dependent on particle size, rate of flow, etc.; only values relative to those of normal adult haemoglobin (A_0) can be obtained with reasonable reproducibility.

Carboxymethyl cellulose is also more suitable for preparative separation of the various haemoglobin components from mixtures, because of its higher (reversible) adsorption capacity. In one case, however, Amberlite IRC-50 is to be preferred, namely in cuvette chromatography, where the different haemoglobin types are recognized by visible separation on the column. Because of the high buffering capacity of the resin (which in gradient elution chromatography is a disadvantage), a pH and cation concentration gradient can be maintained on the column for several hours, simply by passing a buffer solution of constant pH and ionic strength through the resin column, which was originally in the acid form. With this procedure haemoglobin types can be seen as sharp, distinct, zones, moving with different, but very low, rates of displacement.

Comparing chromatographic techniques with other methods employed in haemoglobin differentiation, it can be said that, in general, chromatographic elution curves show much detail, certainly more than electropherograms and at least as much as salting-out diagrams. The advantage of elution chromatography over the salting-out procedure of DERRIEN *et al.* lies in the fact that the different components obtained in the elution procedure are fairly pure and not denatured, and therefore ready for further characterization.

As a method for quantitative estimation, chromatography is comparable to electrophoresis, cuvette chromatography to paper electrophoresis, and CMC elution curves to moving-boundary diagrams. As a routine method, chromatography is more laborious than electrophoresis, especially paper electrophoresis. However, as regards simplicity the cuvette technique comes very close to paper electrophoresis. Paper electrophoresis (or zone electrophoresis on cellulose acetate strips) and the chromatographic cuvette technique as well as a combination of the methods are very suitable for the detection of abnormal haemoglobin in clinical routine analysis, as will be shown in the next part of this review.

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III. The Detection and Characterization of Abnormal Human Haemoglobins by Combining Different Techniques

1. CHARACTERISTICS OF DIFFERENT TYPES OF HUMAN HAEMOGLOBIN

Normal adult haemoglobin

Moving-boundary electrophoretic patterns in general contain one sharp peak; the cathodic mobility in 0.1 *M* phosphate buffer, pH 6.5, is $2.4 \cdot 10^{-5}$ $cm^2\ V^{-1}\ sec^{-1}$; iso-electric point in 0.1 *M* phosphate buffer at pH 6.87.

In *zone electrophoresis* at pH 8.6 on paper or cellulose acetate, or in starch, the main fraction has a mobility similar to that of the β_2-globulin fraction of human serum. In starch electrophoresis, besides the main fraction (A_0) two minor components are seen: one (A_1) migrates slightly more rapidly than A_0, the other (A_2, about 2–3% of the total haemoglobin) moves more slowly and separation from the main fraction is good in starch as well as on cellulose acetate strips. The amount of this A_2 fraction is generally increased in thalassaemia trait to about twice the normal value.

In *column chromatography* on Amberlite IRC-50 besides the main fraction another component is seen, which moves a little more rapidly, this component (designated Hb-a or Hb-A_I) represents about 10% of the total haemoglobin.

pH-gradient elution chromatography on carboxymethyl cellulose gives rise to three peaks: the first (A_1, 10%) has an elution pH of 7.40, the second and main fraction (A_0) has an elution pH of 7.49 and the third fraction (A_2, 1%) is eluted at pH 7.69. The A_2 level is elevated in thalassaemia trait.

The chromatographic fractions A_1, A_0 and A_2 seem to be identical with the corresponding electrophoretical zones A_1, A_0 and A_2, but this has not yet been proved.

Adult oxy-haemoglobin is very rapidly denatured in the *alkali-denaturation test*. Its "half-life" in 0.05 *N* NaOH solution is about 4 sec[17].

Solubility. In the salting-out method of Derrien *et al.*[67] (see p. 140) HbCO-A is precipitated in the range from about 77 to 89 vol. % of 3.5 *M* phosphate[9, 27, 67]. Deoxygenated Hb-A is precipitated in the range 72–84%[27]. The solubility of deoxygenated Hb-A in 2.58 *M* (= 74% of 3.5 *M*) phosphate solution at 25° is 1.39 ± 0.15 g/l as estimated by Itano[36].

From experiments with dinitrophenyl-globin Rhinesmith, Schroeder and Pauling[64] concluded that there are 4 N-terminal amino acid residues in the haemoglobin molecule, all valyl residues.

Foetal haemoglobin (Hb-F)[7]

In *moving-boundary electrophoresis* in 0.1 *M* phosphate buffer, pH 6.5, Hb-F does not separate from Hb-A. Separations are only possible in special buffer solutions, for instance phosphate buffer pH 6.8, ionic strength 0.01[3, 71]. The iso-electric point in 0.1 *M* phosphate buffer is at 6.98 (Hb-A: 6.87).

In *zone electrophoresis* at pH 8.6, Hb-F moves slightly less rapidly towards the

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anode than Hb-A_0. Mixtures of Hb-A and Hb-F behave as a single spot, migrating with intermediate velocity.

In *column chromatography* on Amberlite IRC-50, foetal haemoglobin is more weakly adsorbed than adult haemoglobin. In the cuvette method it is displaced 1.3 times more rapidly than Hb-A. In Amberlite chromatography a subfraction is also seen (Hb-f or Hb-F_I), which moves ahead of the main fraction[2, 55, 62]. In pH-gradient elution chromatography on CMC, Hb-F is eluted a little later than Hb-A_0. The elution pH of Hb-F is 7.58 (Hb-A_0: 7.49).

In the *alkaline denaturation test* HbO_2-F is much more resistant than Hb-A, its "half-life" in 0.05 *N* NaOH solution is about 20 minutes[6]. The alkaline denaturation technique is the best quantitative estimation method for Hb-F.

Absorption spectrum: In the case of Hb-F the so-called "tryptophan absorption maximum" is located at 289.8 mμ, whereas for all other haemoglobins known at present it is situated at 291 mμ. By means of this spectral speciality, isolated HbCO- or HbO_2-F fractions can very easily be identified[3, 43].

In the DERRIEN *salting-out method* HbCO-F is precipitated in the range from 88–95 vol. % of 3.5 *M* phosphate solution, pH 6.5 (HbCO-A: 77–89%). For deoxygenated Hb-F, the range is also a few % higher than normal. The solubility of deoxygenated cord blood haemoglobin in 2.58 *M* phosphate solution is 2.2 $\pm$ 0.2 g/l as estimated by ITANO[36].

The *amino acid composition of Hb-F* differs markedly from that of Hb-A, as estimated by different investigators[26, 72, 73]. A very typical difference is found in the isoleucine content: Hb-A contains 0.32 g of isoleucine per 100 g of haemoglobin, whereas Hb-F contains 1.83 g per 100 g. From hydrolysis experiments with DNP-globin, SCHROEDER AND MATSUDA[74] conclude, that foetal haemoglobin contains 2 N-terminal valyl residues instead of the 4 valyl residues found in normal adult haemoglobin.

Sickle-cell haemoglobin (Hb-S)[58]

Good separation from Hb-A is obtained in *moving-boundary electrophoresis* in 0.1 *M* phosphate buffer, pH 6.5, mobility: $2.9 \cdot 10^{-5}$ $cm^2\ V^{-1}\ sec^{-1}$.

In *zone electrophoresis* separation of Hb-S from Hb-A and Hb-F is also good. It migrates more slowly than the latter components. In electrophoresis in starch or on cellulose acetate strips it moves slightly more rapidly than the Hb-A_2 component.

In the *cuvette method with Amberlite IRC-50* the rate of displacement of Hb-S is 0.7 times that of Hb-A. Separation from Hb-A is good.

In *pH-gradient elution chromatography* on CMC its elution pH is found to be 7.64 (between that of Hb-F and Hb-A_2).

A very characteristic property of sickle-cell haemoglobin is its low *solubility* in the deoxygenated form. In this form it is precipitated in the salting-out method[27] within the range 52–66 vol.% of 3.5 *M* phosphate solution, whereas the precipitation range of the CO-form is almost normal (75–84%). The amino acid composition of Hb-S is almost identical with that of Hb-A[26, 73]. INGRAM, using a combination of

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paper electrophoretic and chromatographic techniques in the investigation of tryptic digestion products of Hb-S and Hb-A concluded that at two places in Hb-S there are valyl residues present instead of the glutamic acid residues found in Hb-A[32].

Haemoglobin C[34]

In *moving-boundary electrophoresis* in 0.1 *M* phosphate buffer, pH 6.5, the mobility of Hb-C has the highest value so far found for human haemoglobin: $3.2 \cdot 10^{-5}$ cm^2 V^{-1} sec^{-1}. The iso-electric point in 0.1 *M* phosphate buffer is 7.30.

In *zone electrophoresis* at pH 8.6 as well as in *chromatography on Amberlite IRC-50*, Hb-C has the lowest rate of migration of all hitherto known haemoglobin types. In the cuvette method it is displaced 0.3 times as fast as Hb-A. Its elution pH in *CMC chromatography* is very high: 7.82. The *precipitation range* of HbCO-C in phosphate solution pH 6.5 is 68–76 vol. % of 3.5 *M* solution, as estimated by HUISMAN *et al.*[27], DERRIEN *et al.* have reported much higher values (83–93%). This difference in results has not yet been explained. According to HUISMAN *et al.*[27] the precipitation range of deoxygenated haemoglobin C falls almost within the normal range. On the other hand, ITANO reports a solubility (3.46 ± 0.41 g/l) in 2.58 *M* phosphate buffer higher than that of Hb-A (1.39 ± 0.15 g/l).

Using the technique mentioned above, INGRAM estimated that Hb-C differs chemically from Hb-A at the same two sites of the molecule as Hb-S; in Hb-C the glutamic acid residues of Hb-A are replaced by lysine residues.

Haemoglobin D[35]

The *electrophoretical properties* of Hb-D are identical with those of sickle-cell haemoglobin (the same mobility in buffer solutions of various kinds and pH's, the same iso-electric point).

In *cuvette chromatography* on Amberlite IRC-50, Hb-D could also not be distinguished from Hb-S.

A characteristic difference between Hb-S and Hb-D is found in the *solubility*, the deoxygenated form of the latter has a precipitation range in phosphate solution of the same order as that of Hb-A[9], whereas deoxygenated Hb-S is precipitated at much lower phosphate concentrations. For distinguishing between Hb-S and Hb-D, the simplified solubility method of ITANO[36] (see p. 142) is very useful.

Haemoglobin E[37]

Electrophoretical characteristics. The mobility in 0.1 *M* phosphate buffer, pH 6.5, is between that of Hb-A and Hb-S, the iso-electric point in 0.1 *M* phosphate buffer is at pH 7.20 (between Hb-S and Hb-C), the mobility in barbital buffer pH 8.6 is also between that of Hb-S and Hb-C, the pH–mobility curves of Hb-S and Hb-E thus cross each other at a pH between 6.5 and 7.2. Electrophoretically Hb-E cannot be distinguished from the normal A_2-fraction.

Chromatography. On Amberlite IRC-50 columns at pH about 6, Hb-E has a rate of displacement between those of Hb-A and Hb-S, it cannot be well separated from

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Hb-A or Hb-S in this way. In pH-gradient elution on CMC columns, the elution pH of Hb-E is 7.68, similar to that of the Hb-A_2 fraction (7.69) and between those of Hb-S and Hb-C.

Amino acid analysis, as performed by STEIN *et al.*[79] revealed no marked differences between Hb-E and Hb-A.

Haemoglobin G[14]

In *electrophoresis* the pH–mobility curve lies between that of Hb-A and Hb-S. In 0.1 *M* cacodylate buffer pH 6.5, Hb-G separates well from Hb-A, no good separation of Hb-G and Hb-S can be obtained under these conditions. In barbital buffer pH 8.6, Hb-G separates well from Hb-S, but very poorly from Hb-A.

In Amberlite *chromatography* with the cuvette method, Hb-G is not separated from Hb-A, although it has a somewhat lower rate of displacement.

Solubility. Deoxygenated Hb-G is more soluble (5 g/l) than Hb-S (0.3 g/l) in 2.24 *M* (= 64% of 3.5 *M*) phosphate solution, but less soluble (0.6 g/l) than deoxygenated Hb-A (1.4 g/l) in 2.58 *M* (= 74% of 3.5 *M*) phosphate solution.

Haemoglobin H[65]

Electrophoretical data. The iso-electric point lies between pH 5.5 and 5.65, thus in contrast to the other Hb types, at pH 6.5 it moves towards the anode (mobility $-1.5 \cdot 10^{-5}$ cm^2 V^{-1} sec^{-1}, differing considerably from that of Hb-A: $2.4 \cdot 10^{-5}$ cm^2 V^{-1} sec^{-1}). At pH 8.6 in 0.1 *M* barbital buffer the difference is much smaller; the mobilities of haemoglobins H and A are then -3.8 and $-3.0 \cdot 10^{-5}$ cm^2 V^{-1} sec^{-1} respectively.

In *Amberlite chromatography*, Hb-H has the highest rate of displacement of the haemoglobins known so far. It separates well from Hb-F[25] which is also fast-moving.

In pH-gradient *CMC chromatography* Hb-H has the lowest elution pH: 6.7–6.9[24].

In the *solubility test* according to ITANO, deoxygenated Hb-H is practically insoluble in 2.24 *M* phosphate solution; after precipitation it cannot be redissolved by dilution of the buffer and oxygenation. Hb-H is also unstable in aqueous solution: a brownish flocculent precipitate is formed on standing.

The *amino acid composition* of Hb-H is different from that of Hb-A; it resembles that of Hb-F[29].

Haemoglobin I[69]

The *pH–mobility curve* and thus also the iso-electric point of Hb-I lie between those of Hb-H and Hb-A. The mobility in 0.1 *M* cacodylate buffer pH 6.5, is $1.7 \cdot 10^{-5}$ cm^2 V^{-1} sec^{-1}. The difference in the mobilities of Hb-A and Hb-I ($0.6–0.7 \cdot 10^{-5}$ cm^2 V^{-1} sec^{-1}) remains practically constant throughout the whole pH–mobility curve. Consequently Hb-I can be well separated from both Hb-A and Hb-H by electrophoresis at pH 6.5, but it is indistinguishable from Hb-H in paper electrophoresis at pH 8.6.

In *Amberlite chromatography*, Hb-I has a rate of displacement between those of Hb-A and Hb-F; mixtures of Hb-A and Hb-I form two well-separated zones[25].

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Haemoglobin J[81]

Between pH 6.5 and pH 9.8, the *pH–mobility curve* of Hb-J lies between those of Hb-A and Hb-I. The difference in mobility between Hb-A and Hb-J is constant in this interval and is $0.3–0.4 \cdot 10^{-5}$ $cm^2 V^{-1} sec^{-1}$. In paper electrophoresis in 0.025 *M* barbital buffer pH 8.6, a good separation of Hb-J and Hb-A can be achieved.

In *Amberlite IRC-50 chromatography* mixtures of Hb-A and Hb-J could not be separated[22]. In pH-gradient elution *chromatography on CMC*, Hb-J behaves very similar to the chromatographic fraction A_1. The elution pH of Hb-J is 7.39 ± 0.01, that of Hb-A_1 is 7.40 ± 0.01[24].

A mixture of deoxygenated Hb-A and Hb-J is significantly more soluble in 2.58 *M* phosphate buffer than Hb-A alone (2.00 ± 0.24 and 1.39 ± 0.15 g/l resp.).

Haemoglobin K[9]

In 0.025 *M* barbital buffer, the *electrophoretic mobility* of Hb-K is somewhat higher than that of Hb-A, but lower than that of Hb-J. It cannot be separated from Hb-A in paper electrophoresis, but a difference can be seen between spots obtained from normal Hb and those from mixtures of Hb-A and Hb-K.

In *chromatography on Amberlite IRC-50*, no difference between Hb-A and Hb-K can be observed.

In the *salting-out method* of DERRIEN *et al.*[68], HbCO–AK-mixtures give curves that are identical with those of HbCO–AD and AS-mixtures[9].

Haemoglobin L[1]

By means of *electrophoretical methods* Hb-L can only be separated from Hb-A at alkaline pH. In starch electrophoresis at pH 8.6, the mobility of Hb-L lies between those of Hb-A and Hb-G.

In *chromatography on Amberlite IRC-50* Hb-L forms a zone, moving with a rate of displacement between those of Hb-S and Hb-C, thus very different from Hb-A. In pH-gradient elution *chromatography on CMC*, the elution pH of Hb-L (7.71) also lies between those of Hb-S and Hb-C.

The *solubility* of Hb-L in phosphate solutions is reported to be normal.

Fig. 20 presents a schematic diagram of the properties of various haemoglobin types in electrophoresis and in Amberlite chromatography. It can be seen from this diagram that the two techniques complement each other in many cases, for instance, where electrophoresis at pH 8.6 fails to distinguish between adult and foetal Hb, or between Hb-G and Hb-K, or between Hb-H and Hb-I, Amberlite chromatography gives very good resolutions. On the other hand, where Amberlite chromatography gives no good separations (Hb-A and Hb-J, Hb-A and Hb-E), these can be achieved by electrophoresis. The combination cuvette chromatography + paper electrophoresis fails to distinguish between Hb-S and Hb-D, both haemoglobins behave identically in electrophoresis as well as in chromatography on Amberlite IRC-50. In this case resort

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must be made to solubility estimations. In all other cases so far known, paper electrophoresis in combination with chromatography on Amberlite cuvettes leads to a qualitative and sometimes a semi-quantitative analysis of an unknown haemoglobin mixture, provided the various fractions are present in amounts above 5%. An abnormal fraction, once detected, can be further characterized by the application of

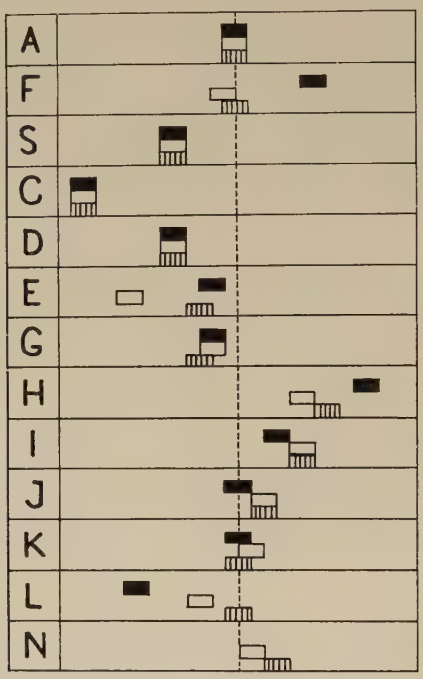

Fig. 20. Schematic comparison of the relative mobilities of different types of human haemoglobin in: Zone electrophoresis at pH 8.6 (□ —→ +), Moving-boundary electrophoresis at pH 6.5 (▥ —← +), Chromatography on IRC-50 at pH about 6.5 (■ →).

alkali denaturation, solubility studies, chromatography on carboxymethyl cellulose, mobility studies in moving-boundary electrophoresis, estimation of amino acid composition.

The application of different techniques is illustrated in the following description of a study of haemoglobins in the case of a family consisting of father, mother, two sons and three daughters[31]:

The haemoglobin of the mother (indicated as Mrs. W) behaved in cuvette chromatography as a tailing zone, with a rate of displacement corresponding to that of normal adult Hb. In paper electrophoresis at pH 8.6 on Whatman No. 3 MM paper, two well-separated zones were observed, one with a velocity equal to that of Hb-A, the other with a velocity somewhere between those of Hb-S and C.

These results indicated the presence of Hb-E besides Hb-A. Additional experiments revealed, that no alkali-resistant fraction was present; moving-boundary

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electrophoresis showed two components, one (62%) with a mobility similar to that of Hb-A, the other (38%) with a mobility similar to that of Hb-E.

The haemoglobin of the father showed no abnormality in paper electrophoresis; in cuvette chromatography a small zone was observed (about 10% of the total Hb) with a rate of displacement similar to that of Hb-F. Alkali-denaturation velocity

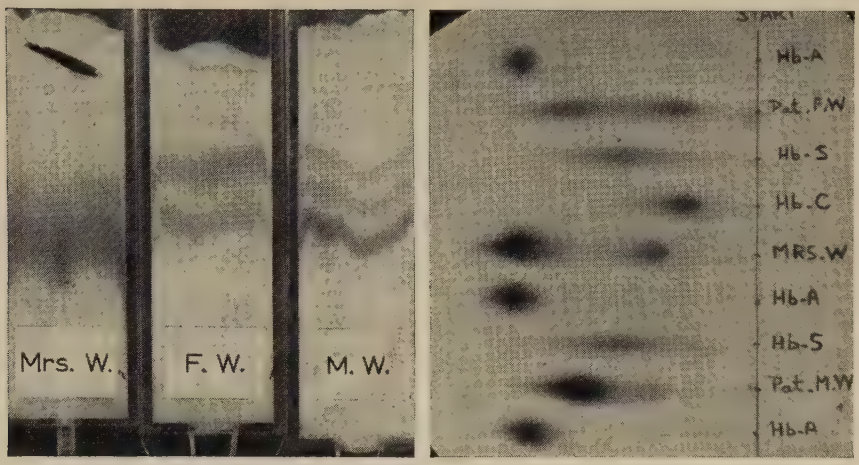

Fig. 21. A combination of cuvette chromatography and zone electrophoresis at pH 8.6 in a study of a family, in which Hb-E as well as thalassaemia occur.

measurements indicated that there was 11% of an alkali-resistant fraction. Thus, the blood of the father contained besides normal adult Hb, 11% of foetal haemoglobin, as in many cases of thalassaemia trait.

Two children exhibited the same haemoglobin pattern as that of the mother, another child showed that of the father.

The fourth child (designated pat. F. W.), a boy of five years, had severe anaemia. Electrophoretic and chromatographic haemoglobin patterns are shown in Fig. 21. In the chromatogram, two zones can be seen, one (58%) with an almost normal velocity, the other (42%) with a velocity like that of foetal Hb. In the electrophoretic pattern, an F-like and an E-like zone were observed. Alkaline-denaturation velocity estimations revealed that 43% alkali-resistant Hb was present, moving-boundary electrophoresis showed that besides a resembling Hb-A component (62%), 38% Hb-E was present. From the results obtained with chromatography and alkaline denaturation it seemed very probable that 42% of the component that resembled Hb-A in electrophoresis, consisted of Hb-F, therefore it was assumed, that 20% of the total Hb consisted of Hb-A. Cuvette chromatography with about 5 mg HbCO and with slow elution velocity resulted in a chromatogram containing three zones, very probably representing Hb-F, Hb-A and Hb-E (in order of decreasing rate of displacement) (Fig. 13, 3, p. 150). The child is an example of a patient suffering from heterozygous thalassaemia Hb-E disease. The fifth child (pat. M. W.) was seven

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months old when examined. Her blood still contained 71% of alkali-resistant haemoglobin, the rest was probably a mixture of Hb-A and Hb-E (see Fig. 21 for the chromatogram and electrophoretic pattern). This child is very probably also heterozygous for thalassaemia and Hb-E.

This family study shows that also in more complicated cases, the abnormal Hb-fractions can be detected and provisionally identified by a routine combination of chromatography and zone electrophoresis. However, it is necessary that an abnormal haemoglobin, once detected, be further characterized with other methods.

2. CONCLUSIVE REMARKS

During recent years, a number of techniques that can be applied on an analytical as well as a preparative scale, have been adopted for the study of abnormal haemoglobins. Zone electrophoresis and chromatography proved to be useful, because both techniques can be applied in mass surveys.

Paper electrophoresis in particular is very extensively employed as a clinical routine method for the detection of abnormal haemoglobins; most of the hitherto known haemoglobins can indeed be detected with paper electrophoresis. Recently, however, a number of abnormal haemoglobins have been discovered, the electrophoretic behaviour of which is very similar to that of normal adult haemoglobin. These abnormal haemoglobins are very easily overlooked if only one technique is applied. Therefore it is suggested that a combination of at least two techniques should invariably be used for the detection of abnormal haemoglobins.

The techniques must be improved because of the growing importance of the evaluation of the so-called "sub-fractions" (in the diagnosis of thalassaemia trait).

It is to be expected that the replacement of the protein-adsorbing paper strips in zone electrophoresis by non-adsorbing material, such as starch and cellulose acetate, and that the introduction of cellulose ion-exchangers in chromatography will contribute much to making the analyses of haemoglobin mixtures more sensitive. Such sensitive methods of analysis are necessary, because further investigations can only be of importance in the study of heredity and haemoglobin synthesis, if performed on very clearly defined haemoglobin samples.

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INORGANIC ADSORPTION AND PRECIPITATION CHROMATOGRAPHY

E. HAYEK

Institute of Inorganic and Analytical Chemistry, University of Innsbruck (Austria)

1. INTRODUCTION

In the field of chromatographic separation of inorganic substances, two methods are frequently and successfully used; these are ion-exchange chromatography and partition chromatography.

The boundaries between these methods are seldom clearly defined in practice owing to the exchange properties of cellulose. The limits are even less well defined in the case of a third chromatographic method which corresponds to the method, widely used in organic chemistry, based on "adsorption" and which is also designated as adsorption chromatography in inorganic chemistry, although under the prevailing conditions the processes may be quite different from those of the applications to organic compounds; exchange and, particularly, precipitation reactions may play a considerable part.

In the present review, the development of this field is fully described and exchange or partition chromatography is mentioned only in so far as doubtful or borderline cases are concerned. Such cases may occur for example in partition chromatography; if a water-miscible solvent such as methanol is used in an aqueous system, it can form an adsorption layer on paper and act as a second liquid phase (see under 2 (b)). For the boundaries of exchange chromatography, see under 2 (f).

2. DEFINITIONS

In the literature many terms are used in different or ambiguous ways, hence it is necessary to define them exactly, especially in relation to the terms used in organic chemistry.

(a) It appears necessary to define even the term "chromatographic process" more closely, namely, as a separation of at least one component of a solution by fixation in a zone on the surface of a solid material from a liquid flowing through the material in a constant direction.

(b) "Adsorption" is used in the following pages to designate the fixation of a dissolved substance on a surface without regard to the mechanism of the process. It is possible to differentiate specifically between molecular adsorption, ionic adsorption (addition of ions to oppositely charged positions on the adsorbent), exchange adsorption (displacement of an ion of the adsorbent by one from the solution) and precipita-

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tion adsorption (reaction of a soluble part of the adsorbent with components of the solution directly on the surface, the solid phases being fixed to the surface).

When *one* solvent phase is used, it may happen that the surface of the adsorbent becomes increasingly covered with a molecular layer which does not correspond to the composition of the homogeneous solvent when this consists of a mixture, *e.g.* water–methanol. The chromatographed material is then increasingly transferred from the state of adsorption on the solid material to the state of solution in this surface layer, just as happens in partition chromatography when two liquid phases are present. This is the borderline case between partition and adsorption chromatography which was mentioned earlier.

(c) The process which follows the addition of the liquid sample to the chromatographic column (or film or fibre) and which is intended to achieve better separation into zones, is known as "development" in organic chromatography. In inorganic chromatography the process is often called "washing apart" and this expression is used in the present review.

(d) In inorganic chromatography a further process is usually necessary, namely, treatment with another solution in order to make the individual zones clearly visible. This is usually called the "development" but the expression "staining" is employed here in order to avoid misunderstandings.

(e) "Elution"—the process which involves the displacement of the adsorbate by the solvent—plays a great part in organic chromatography, but in inorganic chromatography the process cannot always be carried out in an analogous way. For example, washing with water may cause hydrolysis, or displacement by other dissolved ions, especially hydrogen ions, may occur. In any case, the mechanism is by no means always unambiguously clear. In the present review "elution" is used quite generally to designate washing with water, a solvent or some other reagent solution. No consideration is given to whether the removal of the adsorbate zone thus obtained depends on "displacement" alone or on a chemical reaction.

(f) It is particularly important to clarify the much-used terms "exchange" and, especially, "ion-exchange". The following scheme of terminology seems to be appropriate.

Any reaction between a solid material constructed of ions and ions from the surrounding solution according to the equation:

$$AB_{solid} + C^- = AC_{solid} + B^- \qquad (1)$$

or an analogous cation-exchange, can be considered as a *formal ion-exchange*.

This formal stipulation can, however, be fulfilled in various ways:

(1) A *constant structure* exchange occurs in the case of high-polymer, and thus insoluble, zeolites and synthetic resins; in the former case with cations, and in the latter, cations or anions according to the structure of the resin. The transformation of ion solutions with difficultly soluble substances can be placed in this category in so far as no alteration in the lattice structure is produced, *e.g.*

$$Ca_5(PO_4)_3OH + F^- = Ca_5(PO_4)_3F + OH^-$$

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Such processes can be designated as ideal ion-exchange, for they involve a direct change of place of the ions at a fixed position of the lattice.

(2) A process which likewise corresponds to the general formal exchange equation, but in which the structure of the adsorbent is modified, is called a *variable structure* exchange. An example is provided when cubic silver chloride is converted to hexagonal silver bromide by reaction with bromide ions in the surrounding solution, also if the exchange is confined to the surface.

Of special interest for the present purpose is the reaction of aluminium oxide with an aqueous metal salt solution, *e.g.* according to the equation:

$$Al(OH)_3 + MX_2 = Al(OH)_2X + M(OH)X \qquad (2)$$

This reaction may lead to the formation of two solid phases on the surface of the adsorbent. Analytically, the formal exchange reaction appears as a fixation of the whole compound MX_2 on the adsorbent. Such processes are frequently designated by the collective term "equivalent adsorption" (of cation and anion), but it would be more meaningful in this case to ascribe them to the essential basic reaction of precipitation.

If a reaction product is soluble, the reaction may appear analytically as an exange between a cation and an anion. Thus either the aluminium or an added metal ion, *e.g.* thallium (I), can go into solution (*e.g.* as the hydroxide-chloride or the hydroxide, respectively); it is adsorbed only further down the column and can then be eluted. Meanwhile the accompanying anion, especially if it is sulphate, remains much more firmly attached to the aluminium oxide. In the former case precipitation of the metal hydroxide-chloride occurs on the column, and in the latter it is a case of a variable structure, and thus only formal, exchange of anions.

The surface of the adsorbent certainly plays an essential role in precipitation reactions of this type, as in all "topochemical processess".

3. DEVELOPMENT AND CLASSIFICATION

Some chronological notes on the development of inorganic chromatography in general, as well as of special fields, are given below.

The first type of chromatography to be used for inorganic salt solutions is certainly the "filtration method". EICHHORN[20] applied this technique with natural zeolites for hardness of water almost exactly 100 years ago, and it was later used with the synthetic materials, permutites, developed by GANS[42]. It is also possible to trace individual studies on paper chromatography just as far back; as early as 1861, SCHÖNBEIN[153] observed the different distances at which salts remain in relation to the water front in paper strips.

Precipitation chromatography was certainly first introduced when impregnated paper was used in the technique of spot tests, especially for the detection of two ions in presence of each other. FEIGL[27] described the first case of this type in 1930. Since then, spot tests have developed[26] into a well-known and independent field and cannot be considered further here.

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The problems involved in the processes used for the chromatographic separation of inorganic ions were first investigated by SCHWAB[154] with aluminium oxide, which was applied to inorganic compounds after it had proved its excellent properties for organic chromatography. The explanation of the mechanisms has, however, been found extremely difficult because of the superposition of different processes, and the results are much less favourable than in organic chemistry.

SCHWAB himself suggested or discussed several foundations for the course of the processes.

1. The permutite-like displacement of sodium ions by the chromatographed cations, *i.e.* exchange adsorption[155].

2. An analogous exchange adsorption of anions[157].

3. The formation of basic double aluminates[161–163,165]. He rejected the precipitation of hydroxides[161] or basic salts[165] and the exchange of metals with Al^{3+}[155].

In the numerous publications of the succeeding 20 years, "exchange", "adsorption" and "precipitation" have been used in different combinations, and often contradictorily, to describe the reasons for the fixation of inorganic ions, especially on aluminium oxide columns. Even in the latest books and review literature[8,76,141,179,180,209] no clear classification of inorganic chromatography in these groups is to be found. In the ensuring pages the discussion is weighted in favour of the causes underlying the technique as opposed to an evaluation of its use for qualitative and quantitative purposes, for these have perhaps not fully lived up to expectations.

As mentioned above, the majority of investigations has been made with alumina, hence this must be discussed first in relation to the three most important attempted explanations, *i.e.* exchange, (physical) adsorption and (hydrolytic) precipitation; then the behaviour of anions must be considered, for this is also important in the separation of cations. Studies involving the impregnation of various carriers can be classified together in a group forming a transition to solid adsorbents which themselves react with salt solutions. The technique has been widely used for quantitative purposes, especially on the micro scale, and it has also been applied in the purification of salt solutions. Special methods have been evolved by the application of complexing agents and radioactive indicators, as well as for different groups of elements.

In the following pages, the various studies are discussed essentially in chronological order.

4. PARTICULAR INVESTIGATIONS

(*a*) *Chromatography of cations on alumina from the viewpoint of ion-exchange*

In his first publications SCHWAB[154,155] considered the fixation of cations as being essentially due to the exchange of heavy metal ions with sodium ions which could then be detected in the filtrate. Most alumina preparations for chromatographic purposes contain sodium as carbonate or aluminate from the production method. According to SCHWAB, sodium can be replaced by the metal as in the equation:

$$>Al\text{–}O\text{–}Na + M^{n+}/n = >Al\text{–}O\text{–}M/_n + Na^{+} \qquad (3)$$

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so that aggregates of the spinel type are formed. Investigations of binary mixtures showed that cations which could be chromatographed from solutions (in absence of complex formation) formed the following sequence:

$$\begin{matrix} & Cr^{3+} & & & & & Co^{2+} \\ H^+, & Fe^{3+}, & UO_2^{2+}, & Pb^{2+}, & Al^{3+}, & Cu^{2+}, & Ag^+, & Zn^{2+}, & Ni^{2+}, & Tl^+, & Mn^{2+} \\ & Hg^{2+} & & & & & & & Cd^{2+} & & \\ & & & & & & & & Fe^{2+} & & \end{matrix}$$

FLOOD[34] assumed this same type of exchange adsorption for his studies of alumina precipitated on paper, and VENTURELLO[204a] also considered the process to be a possible explanation. LINDNER[92–97] likewise mentioned exchange adsorption in his work on aluminium oxide with radioactive indicators. HESSE[68] formulated the substitution of sodium ions by silver ions in an analogous way. He also referred to the fact that acids (anions) are more readily adsorbed on alumina from acidic media whereas bases (cations) are better adsorbed from basic media; he considered ion-exchange to be the best explanation of this phenomenon.

SCHWAB[154] had mentioned the fact that cation zones end up in close proximity to each other. JACOBS[73] ascribed this to the complicating effect of simultaneous anion adsorption and explained the adsorption as ion-exchange and molecular fixation. However, he indicated in this connexion that cations, with the exception of hydrogen ions, are unable to displace other adsorbed cations to any great extent.

Similarly, GAPON[43,45] explained the fixation of cations on alumina as an exchange reaction, in so far as anions are not retained simultaneously. ROBINSON[142] believed that only ion-exchange was a valid explanation in the case of cations, as against the (physical) adsorption of the molecule.

FISCHER[29] established by static methods that an exchange of sodium ions with an equivalent amount of the cation can occur, from which he inferred the chromatographic mechanism; but it remains uncertain whether it is a matter of precipitation or permutite exchange. Besides, an equivalent adsorption of the cation and anion of the salt occurs. UMLAND AND FISCHER[194,195] later developed the idea that the primary process of adsorption of ionised salts from an aqueous solution depends on a coupled exchange of cations on one side and anions on the other side with the participation of hydrogen and hydroxyl ions as had already been suggested by SACCONI[145,149]. The results obtained by the static investigations of UMLAND[197–199] have been thoroughly discussed and developed into a formal ion-exchange theory for the adsorption of electrolytes.

D'ANS[16,17] considered that not only sodium ions but also hydrogen ions could exchange with metal ions, and likewise utilized studies with suspensions as part of the basis of his discussions. With regard to this, HAYEK[66] pointed out that conclusions reached from static investigations can only be applied with reservations to the situation which exists on a column. The pH values set up in a column do not correspond to those of a static equilibrium, and much greater extremes of pH can be measured. Besides, various well-known facts show that the sodium content of alumina, which is the formal

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cause of the ion-exchange according to equation (3), can be liberated by decomposition in the following steps:

$$>Al\text{–}O\text{–}Na + H_2O = >Al\text{–}OH + NaOH \quad (4)$$

$$NaOH + MeX_2 = Me(OH)X + NaX \quad (5)$$

The hydrolysis of sodium aluminate according to equation (4) results in the pH of an alumina suspension increasing to a value of 9–10 in the case of the usual Al_2O_3 preparations (HESSE[69]). Under these conditions no exchange of heavy metal ions could occur on a column, for only precipitation of basic salts or hydroxides would be possible.

In order to avoid the complications introduced by the sodium content of alumina, even early investigations were carried out with *pure aluminium oxide* which was prepared by precipitation with ammonia or from amalgam. SCHWAB[155] found that such a preparation in fact gave the same sequence of cations but longer zones and a definite possibility of elution. VENTURELLO[205] investigated the reflection spectrum of adsorbed copper sulphate and found that it differed little from that of the pure salt; he therefore concluded that no chemical reaction occurred. In contrast, when an adsorbent containing sodium was used, SCHWAB[162] obtained spectra lying between those of the ion itself and the hydroxide, which corresponded to that of the basic salt. He concluded, however, that the combination state should be compared to the basic double salts, *e.g.* perhaps "basic aluminate-nitrates or -sulphates", rather than to aluminates. The replacement of sodium by a heavy metal ion bound to a hydroxyl group and an equivalent of anion, *e.g.* nitrate, can be interpreted in this way.

FRICKE[40], SACCONI[149] and UMLAND[195] established that the metal salt causes the appearance of aluminium ions in the solution,which would indicate a formal exchange. Studies carried out by GRASSHOF[57] with alkali-free aluminium oxide do not agree with other investigations on the pure oxide, but the results were later[58] explained by the calcium content of the preparation.

FRICKE[39] is firmly of the opinion that the fixation of iron(III), copper(II) and cobalt(II) on pure alumina takes place by an exchange of the salt anions with the hydroxyl groups of the adsorbent, so that the sequence corresponds to the increasing basic strength of the heavy metal bases.

According to FISCHER[59], copper(II) and chloride ions are retained on sodium-free alumina in completely equivalent amounts (in static studies), whereas with sodium-containing preparations the copper cation remains in excess. In the former case corresponding to equation (2) the adsorbate can be partly eluted[29]; this was explained by HAYEK[66] as being due to a reversible equilibrium:

$$Al(OH)_3 + MX_2 = Al(OH)_2X + M(OH)X$$

which can be displaced from right to left at greater dilutions. Actually each basic salt is stable only in presence of a certain minimum amount of the neutral salt and unless this is attained, the basic salt hydrolyses to form MX_2 and $M(OH)_2$. The latter must react further with $Al(OH)_2X$ to yield $M(OH)X$ so that MX_2 can be largely reformed. If the equilibrium is displaced to the right for any reason, then the basic

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salt is produced in larger amounts. SCHÄFER[151] proved this by X-ray analysis in the case of $Cu_2(OH)_3Cl$.

(*b*) *Chromatography of cations on alumina from the viewpoint of (physical) adsorption of ions and molecules*

SCHWAB[155] established that the behaviour of mercuric chloride on an alumina column is basically different from that of other mercury salts and other heavy metal salts; thus on washing apart the salt either disappears rapidly or is retained much further down the column. However, SCHWAB did not draw any conclusions from his findings.

VENTURELLO[201–206] ascribed the chromatographic fixation of all cations, at least in the initial stages, to the formation of an electrical double layer. The stability of the linkage is dependent on the charge, size and polarizability of the ions. Only in special cases can "specific affinities" outweigh these forces. A gradual loss of water of hydration when the temperature is raised increases the stability of the fixation and its irreversible character.

JACOBS[73] considered the fixation of cations, in so far as it proceeds simultaneously with anions, to be a molecular adsorption. The magnitude of this equivalent adsorption of cations and anions runs in parallel with the covalent character of the salts. SHIBATA[176] confirmed the cation sequence found by SCHWAB, but explained it as being dependent on the radius of the hydrated ions and on their charge.

FISCHER[29,30,32] ascribed to electrostatic forces that part of the cation adsorption which takes place with equivalent amounts of anions and can be eluted. His investigations were of course carried out essentially not on a column but statically. FISCHER, SCHÄFER AND NEUGEBAUER[41,120,150] established that physical adsorption (= equivalent adsorption) occurs, especially on an alumina column prepared with acid; but they left open the question whether molecules, single ions or ion pairs are adsorbed. They consider the possibility of elution as essential. NEUGEBAUER AND SCHÄFER[120] reported the successful separation of the alkali metals on alkali-free alumina (the Woelm product which contains calcium); the metals were eluted with pure water and their amounts determined by conductivity measurements. Manganese, nickel, cadmium and mercury, were also separated from each other from binary mixtures on a column of acid alumina. The zones appeared to be separated by cation-free zones contrary to what happens on a sodium-containing column.

ERLENMEYER[23] has discussed some of SCHWAB's studies on cations; he suggested the possibility that the oxygen atoms of the alumina participate in the reaction with the formation of hydrogen bridges.

(*c*) *Chromatography of cations on alumina from the viewpoint of hydrolysis and precipitation*

In his initial work, SCHWAB[155] emphasized the exchange of sodium ions with the metal and indicated that other adsorbents such as zinc oxide or magnesium oxide are

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unsuitable owing to their powerful precipitating properties. However, SCHWAB[161] rejected the possibility of hydroxide precipitation on the basis that the sequence on the column does not correspond to the solubilities. The formation of basic salts was also rejected, for HAYEK[65a] had shown that their formation is strongly dependent on the anion present, and this effect is not noticeable in chromatographic studies. HAYEK[66] however called attention to the fact that this argument is not valid because the same anion has a similar effect on all the cations involved, that is, *e.g.* all basic phosphates are more resistant to hydrolysis than the sulphates, which are again more resistant than the nitrates. Thus the addition of anions affects all the cations present in much the same way.

Since the absorption spectra of the adsorbates lie between those of the salt solution and the hydroxide, SCHWAB later[162] came to the conclusion that it is a matter of the formation of basic double salts; he mentioned for example basic aluminate-nitrates and -sulphates. On a literal interpretation, this hypothesis appears somewhat forced because aluminates are not stable under the prevailing conditions of pH (between 5 and 9). It becomes more reasonable if basic aluminium salt is substituted for basic aluminate. Nevertheless, this hypothesis is opposed to an opinion expressed by SIEWERT[177] that carbonates are in fact precipitated, which was later generally accepted by both groups of workers[163]. SIEWERT alone in fact put forward the idea[178] that the cations are fixed by an exchange of the hydroxyl groups of the alumina with the anions of the salt. He considered that to this extent a formation of basic salts or hydroxides can take place and thus produce a precipitation.

JACOBS[73] rejected the idea of a hydrolytic adsorption according to the equation:

$$Me^{++} + H_2O = MeOH^+ + H^+ \qquad (6)$$

because no increase in the acidity of the solution owing to the adsorption could be established. Yet the author appears to realize that the hydrogen ion is well qualified to displace other cations on the column and may thus be rendered inactive.

KUBLI[87] was also interested in the separation of cations; he considered it to be due to precipitation of metal carbonates or hydroxides rather than to exchange processes such as govern the chromatography of anions.

The expression "hydrolytic adsorption" was also used by FRICKE[39] to describe the reaction between potassium chloride and aluminium hydroxide, which results in the pH increasing to more than 9 by a formal ion-exchange according to the equation:

$$Al(OH)_3 + KCl = Al(OH)_2Cl^{\Box}_{\Box} + KOH \qquad (7)$$

SACCONI[145–149] found that the hydrolytic adsorption of cations is promoted by the buffering action of alumina. The degree of hydrolysis of salt solutions is known to run parallel to the sequence of cations on alumina[149]. The water of hydration of the ions is an essential factor in the adsorption (*cf.* VENTURELLO[201–206]), the polarized water molecule being replaced successively by hydroxyl ions according to the equation:

$$6[M(H_2O)_6]^{2+} + Al_2O_3 + 9H_2O = 6[M(H_2O)_5OH]^+ + 2[Al(H_2O)_6]^{3+} \qquad (8)$$

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The $[Al(H_2O)_6]^{3+}$ ion produced is then further hydrolysed, *e.g.* to $[Al(OH)_2]^+$ and hydrogen ion. In this connexion it should be noted[66] that only hydroxylated cations of aluminium are stable and that the ion $[Al(H_2O)_6]^{3+}$ cannot be formed at that stage.

The problems involved in the precipitation have been thoroughly analyzed by SCHÄFER[150,151] who concluded that, for example, cupric chloride is precipitated on pure alumina as the basic chloride:

$$Al_2O_3 + 3H_2O + 4CuCl_2 = 2Cu_2(OH)_3Cl + 2AlCl_3 \quad (9)$$

in addition to a physical (equivalent) adsorption. (As before, it must be remembered that aluminium chloride is unstable in presence of the basic copper salt and a basic ion such as $Al(OH)_2^+$ is formed[66].) Aluminium oxide behaves as a weak solid base in the same way as other stronger solid bases, *e.g.* magnesium oxide or the anion-exchange resin Wofatit M, etc.

UMLAND[195] considered that precipitation as described by SCHÄFER is feasible provided that, in the exchange of anion and cation thus required, the anion-exchange is considerably faster so that hydroxyl ions are produced in excess.

The increase of the covering layer on alumina with increase in temperature was first established by VENTURELLO and was studied more extensively by HEINRICH[67]. The phenomenon indicates a process of hydrolysis rather than of physical adsorption.

YASUNAGA[210] argued from the special behaviour of mercuric chloride and from pH measurements that a precipitation process is the general cause of the chromatographic fixation of cations. SPECKER[181] found that the surface activity of alumina runs in parallel with its solubility in fluoride solutions, which shows the importance of the hydroxyl groups on the surface in the adsorption process.

HAYEK[64,66] mentioned that the same sequence of cations is found on aluminium oxide, glass powder and other adsorbents which increase the pH value; he concluded that the cause of fixation is a precipitation conditioned by pH.

D'ANS[17] expressed the opinion that precipitation of basic salt can occur in addition to ion-exchange when the alumina used has not been washed free of sodium ions. Later however, D'ANS[18] turned against the hypothesis of precipitation, because its existence appeared impossible according to the phase rule. On the other hand, HAYEK[66] argued that the phase rule cannot be applied to the situation which exists on the column, for it is not a static equilibrium. In the same paper, he discussed various experimental data from the scattered literature and some of his own studies, which supported the occurrence of precipitation on the alumina; in particular, the pH values found on the column are more extreme than can be measured in static examinations of the adsorbents[69]. Moreover, HAYEK stressed the widely ignored fact that basic salts of heavy metals are only stable in the acidic pH range so that lower pH values provide no argument against the formation of such precipitates.

NODDACK[121] thoroughly discussed the various observations on the reasons for the fixation of cations on alumina columns, and decided in favour of hydrolytic adsorption and formation of basic salts. A far-reaching parallelism between the degrees of hydrolysis of the salt solutions and their sequence on the column was likewise established by

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SACCONI[149]. The importance of alkali addition in order to improve the precipitation by increasing the pH value was stressed, and an extension of the cation series was described.

(d) The use of alumina on carrier materials

The first investigations of this type were made, as mentioned above, by FLOOD[34–38], who used aluminium hydroxide precipitated on paper. GOTO[56] also used this material; he stained the adsorbed cations with oxine and improved the sensitivity of the detection by using ultraviolet fluorescence. This adsorbent was also used by IIJIMA[72] and HOPF[71] in semiquantitative studies. Both these workers used organic reagents for staining, as did OKA[124–127] and MURATA[109–114] who also obtained quantitative results (see below). VANYARKO[200], as well as ZOLOTAVIN[211], developed numerous separations of pairs of metals.

BEZUGLYI[6] pronounced the results obtained with paper impregnated with aluminium hydroxide to be precipitation chromatography.

(e) Effect of anions on cation adsorption on alumina

SCHWAB[157] found that the sequence of the cations on the column is independent of the anion. The type of anion present causes an alteration in the lengths of the zones under certain conditions; in particular, sulphate has a strong shortening effect in comparison to nitrate and chloride. The addition of all alkali salts can also cause shortening of the zones. This can be explained in different ways: NODDACK[121] and UMLAND[195] referred to the increase in alkalinity according to equation (7), and HAYEK[63] to the effect of the increase in anion concentration on the solubility of the basic salt.

JACOBS[73] found that when copper(II), nitrate and sulphate ions were simultaneously present in the added solution, the copper was retained in an upper sulphate-containing zone and in a nitrate-containing zone lying immediately below it. It has been found repeatedly (GAPON[49], UMLAND[197]) that cations which are introduced to the column as their sulphates are more difficult to elute than when they are applied as chloride or nitrate solutions. GAPON[47] found that the amount of anion which is bound together with the cation is related to the chromatographic sequence of the anions.

FRICKE[39] stressed that the exchange of anions with hydroxyl groups on the alumina is essential for the fixation of cations, and SIEWERT[178] explained in a similar way the binding of cations by the precipitation of basic salts. In addition, the views of UMLAND AND FISCHER[194,195] on coupled ion-exchange (see under 4 (a), p. 174) support the significance of the effect of anions on the fixation of cations.

HAYEK[63] showed that anions which form compounds more difficultly soluble than the corresponding hydroxyl compounds are well qualified to affect the sequence of the cations. Thus in presence of phosphate, silver ions are fixed higher up on the alumina column, while sulphide and ferrocyanide ions have a still more extensive effect on the cation order (see under 4 (g), pp. 181, 182).

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(*f*) *Anion adsorption on alumina and other metallic oxides*

SCHWAB[157] was the first to establish that alumina columns are particularly well qualified to retain anions in a chromatographic sequence if they undergo treatment with acid. He found the following sequence of anions:

$$OH^-,\ PO_4^{3-},\ F^-,\ \begin{matrix}Fe(CN)_6^{4-}\\ CrO_4^{2-}\end{matrix},\ SO_4^{2-},\ \begin{matrix}Fe(CN)_6^{3-}\\ Cr_2O_7^{2-}\end{matrix},\ Cl^-,\ NO_3^-,\ MnO_4^-$$

The favoured position at the beginning of the series is taken by the hydroxide ion, as it was taken by the hydrogen ion in the cation series; this agrees with the buffering action of alumina for both ions. By treatment with the strong acids of the anions perchlorate and nitrate, which appear at the end of the sequence, the column can be made suitable for the adsorption of anions which precede them in the sequence. The process is designed as exchange adsorption. FLOOD[34] confirmed the sequence and the mechanisms using paper impregnated with aluminium hydroxide and treated with acid.

KUBLI[87] also acknowledged the anion exchange and spoke of "alumina-permutite", in contrast to the precipitation chromatography of cations. He found, however, that the sequence of anions on the alumina column corresponds to the order of the solubilities of the basic aluminium salts. Again, as in the detailed discussion on p. 172, it is a case of formal ion-exchange.

KÜHN[88] completed the sequence, and more exact investigations on it were carried out by GAPON[47] and MURATA[109–114], the latter using impregnated paper. D'ANS[17] found that both sulphate and copper ions were adsorbed on an acid column from a cupric sulphate solution, whereas only chloride was adsorbed from a cupric chloride solution. He ascribed this to the divalence of the anion and cation.

Using aluminium oxide on paper, MURATA[114] investigated the effect of pH on the zone widths of acids which form polyvalent anions; the effect varied widely.

HAYEK[65] compared the anion sequence on aluminium oxide with that on zinc oxide, lead oxide, La_2O_3 and Bi_2O_3; the sequences found were completely different and ran largely in parallel with the insolubilities of the corresponding metal salts. HAYEK therefore concluded that surface reactions in the form of precipitations take place, although they appear formally as exchanges. He carried out comparative studies on suspensions and columns prepared with acid-formed oxides, and found that more extreme pH values occurred in the columns; thus, suspension studies do not provide reliable evidence on the conditions obtaining in column chromatography.

(*g*) *Other inorganic adsorbents*

SCHWAB[155] has shown that several oxides, as well as barium sulphate and glass powder, are too inactive for cation chromatography, whereas zinc oxide and magnesium oxide are too strongly basic; but floridin, silica gel and calcium phosphate are suitable. However, he did not give any data on the sequence of the ions for these materials. FLOOD[34] used paper impregnated with chromic hydroxide and obtained results which were at least as satisfactory as those obtained with alumina.

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BACH[2] achieved the separation of copper(II) and cadmium(II) and other cations on zinc sulphide as adsorbent whereas zinc ferrocyanide did not give good results. KUTZELNIGG[89] also separated various metals, especially noble metals, on a zinc sulphide column; he described his method as a "filtration procedure".

MILICEVIC[102] used a lead chloride column for the separation of bromide and iodide, as well as a column of zinc dust for the separation of silver, copper and lead ions by reduction in zones; he called his method "chemigraphy". KOMLEV[80] and TEIGE[190] used a layer of silver sulphate on a carrier for the separation of halides.

SEN[166–170] carried out various cation separations on pencils of writing chalk, which consists of calcium carbonate and calcium sulphate and essentially functions hydrolytically.

GAPON[43] ascribed the separations of various cations on barium aluminate and zinc oxide to exchange. On the other hand, he spoke of precipitation chromatography when he used columns of different carriers (alumina, silica) impregnated with various precipitating reagents such as sodium silicate, potassium iodide, silver sulphate, etc.[48,50,52]. CETINI[12,13] utilized cellulose and inorganic carriers, which he impregnated with potassium hydrogen antimonate, sodium aluminate and other solutions, for the separation of numerous cations. MILONE[103–108] separated cations on basic silica and drew up a sequence which is analogous to that of SCHWAB. Gelatin gel mixed with arsenite, borate, etc., was also studied by MILONE[107]; this should facilitate the subsequent mechanical separation of the zones. KOPYLOVA[81] used ferrocyanide on alumina for the quantitative determination of iron in presence of cobalt and manganese.

HAYEK[63] showed that alumina in the presence of phosphate gives the same cation sequence as in its absence, with the exception of silver which is fixed higher up owing to the insolubility of its phosphate. This effect is even more marked on a column of calcium phosphate, which otherwise yields the same cation arrangement as alumina. Completely different sequences are obtained with calcium-potassium ferrocyanide and zinc sulphide where the corresponding insoluble salts are produced and can be recognized from their colours. With regard to the behaviour of various metallic oxides with anions, see the paper by HAYEK[65] as well as p. 181.

UMLAND[196] used silica gel for the separation of cations and found cation-exchange and adsorption with the formation of surface compounds, which appears to be similar to the precipitation of hydroxides and basic salts.

KRAUS[84–86] employed zirconium oxide and other metallic oxides after pretreatment with bases or acids for the chromatographic separation of cations or anions, and zirconium phosphate for the separation of cations; ammonium chloride could be used as the eluant. He assumed exchange processes as the bases of these reactions, but precipitation reactions also appear to be possible.

(*h*) *Organic adsorbents*

ERLENMEYER[23–25] was the first to utilize organic precipitating reagents for column chromatography; he used particularly hydroxyquinoline and violuric acid on kiesel-

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guhr or starch. A parallelism exists between the sequence of the ions and the solubilities of their corresponding salts. Violuric acid is suitable for the separation of the alkaline earth and alkali metals. ROBINSON[143,144] worked with the same material, extended the cation series and carried out quantitative determinations (see below).

Other organic precipitants, such as benzoinoxime, were tested by HOPF[71]. SHEMYAKIN[172–175] used naphthoquinoline and cupferron and separated, in some instances, by elution with thiocyanate. BACH[2–4] employed nitrosonaphthol, dimethylglyoxime, dithizone and other organic reagents as the column material. BURRIEL-MARTI[9] determined nickel by means of dithizone, and DEAN[19] cobalt by means of nitroso-R salt.

NAGAI[115–118] worked with an oxine column with and without solvent mixtures, and precipitated iron as ferric hydroxide between the oxinates of cobalt and zinc. Paper impregnated with inorganic and organic precipitants was applied by VYAKHIREV[208] in qualitative chromatography. VANYARKO[20] carried out numerous separations of metal pairs with oxine. Other investigations with organic reagents are described below in section (i).

(*i*) *Quantitative determinations and trace analysis*

It has been shown repeatedly that the relative zone lengths on alumina do not give a reliable indication of the proportional amounts of ions and in general, no complete separations can be obtained on this material[32,73,87] unless particular provisions are made[45,78,100,175,182–184]. The use of the above-mentioned precipitating reagents usually gives better separations[25,72,102]. It has frequently been recommended to carry out the usual group separations first[7,28,133,158,159,164,210].

Different column materials are most suitable for the determination of small amounts of *individual* types of ions. Even SCHWAB[155] could detect iron(III) and copper(II) down to 1 μg, and he later[160] lowered the detection limit of iron to 0.01 μg and of copper to 0.05 μg in the separation of iron and copper in the ratio of 1:1 with up to 0.1 μg of each. VENTURELLO[104] determined micro amounts of magnesium on alumina, and PINTEROVIC[139] determined traces of iron(III) in sodium chloride. LECOQ[90] analyzed lead(II) in water, and PFAU[138] minute amounts of copper. BEAUCOURT[5b] detected iron(III) as Prussian blue in the presence of 10,000-fold amounts of chromium or cobalt. MEINHARD[101] lowered the limits of detection by applying alumina on glass slides. TANAKA[186–189] detected as little as 0.001 μg of cobalt and nickel on alumina by staining with ammonium sulphide or dithizone. BURRIEL-MARTI[9] was able to detect 1 part of nickel in 10,000-fold amounts of cobalt on dimethylglyoxime (with calcium carbonate as carrier). ROBINSON[143,144] used hydroxyquinoline on starch columns for the quantitative determination of zinc in Cu–Ni–Zn alloys and silver in Cu–Ni–Ag alloys.

SHEMYAKIN[174] detected traces of iron in sulphuric acid on silica. BACH[3] used alumina with nitroso-β-naphthol for cobalt in dilutions of 10^{-9}. ASHIZAWA[1] detected 0.005 μg of palladium with dithizone. KEMULA[77,78] eluted the adsorbate and determ-

ined cobalt in presence of 100-fold amounts of copper by polarography on the micro-scale. NYDAHL[123] determined sulphur in steel as sulphate on alumina. KHOKLOVA[79] and YASUNAGA[210] detected traces of metals in pharmaceutical preparations. KOMLEV[80] determined small amounts of halides on silver sulphate columns.

BALLCZO[5a] concentrated strontium on an alkali-treated alumina column for a microdetermination. He also separated barium from strontium on a column pretreated with nitric acid; for 250 μg amounts the error was $\pm$ 1%[5]. OSHCHAPOVSKI[136,137] determined minute amounts of nickel in presence of a large excess of copper, cobalt or iron on column materials treated with dimethylglyoxime. MURATA[112] evaluated the surface area of spots on impregnated paper for the determination of iron in amounts of 10–100 μg; even 1 μg could be analyzed by reflection spectrocopy. TROITSKII[193] determined several metals in amounts of 0.5–1 μg per litre by means of precipitating reagents on paper.

(*j*) *Chromatographic purification of salt solutions*

Alumina is very suitable for the purification of metallic, and particularly zinc, salt solutions to be used in the production of phosphorescent materials[191]. For this purpose, SHIKORE[152] also used carriers impregnated with organic precipitants for the removal of nickel and cobalt. LISTER[99] lowered the iron contamination of zinc, cadmium and bismuth salts to less than 2 p.p.m. in this way.

GAPON[54,55] used a combined purification with alumina, zinc oxide and zinc sulphide in the preparation of phosphorescent substances, whereas GURVICH[59] applied dimethylglyoxime for this purpose.

FISCHER[33] was able to decrease the rare earths content of yttrium to less than $2 \cdot 10^{-3}$%.

(*k*) *Application of complex solutions*

Solutions of complexes frequently prove very suitable for separations. The complex formation, of course, often prevents the hydrolytic separation of a part or all of the cations, hence the arrangement of the sequence of adsorption is altered correspondingly. The mechanism of the fixation of metals which are bound in complexes is not clear.

SCHWAB[155] found that the sequence of the cations was completely changed after they had been converted to ammines or tartrate complexes. Elements such as arsenic and antimony are completely and immediately hydrolysed when they are placed in an alumina column as their chlorides, and can generally be chromatographed only in complexed forms, particularly as tartrates. ERÄMETSÄ[21,22] likewise used tartrate, as well as citrate, in order to complex the rare earths on alumina. PINTEROVIC[140] separated arsenic, antimony, bismuth and tin as their tartrates, as did KARSCHULIN[75].

KORENMAN[83] described a microdetection of cadmium by means of a cyanide complex solution on silica.

TIKHOMIROFF[192] separated tantalum from niobium on an alumina column by

elution with ammonium oxalate. SENYAVIN[171] used elution with various complexing agents in order to establish the stability of complexes, and GURVICH[60] made similar comparisons with dimethylglyoxime complexes.

LISTER[98] compared the sequences obtained when the complexing agents, ammonia and tartrate, are added with those obtained from pure aqueous solutions and dioxane-containing solutions. All the sequences appeared to differ and conclusions could be drawn about the degrees of dissociation of the salts. JANKOW[74] utilized various complex formations to augment the separative faculties of the column; thus thiocyanate and fluoride were used in the case of iron. OKAC[128,129] worked with solutions of complex thiosalts and cyanide on impregnated paper and used ring chromatography for these separations.

(*l*) *Radiochemical methods*

The use of radio-indicators with the alumina column was started by LINDNER[92] who applied radio-active lead, bismuth, barium and radium. His later work deals with the separation of the rare earths[93–97], but his results did not attain the level of those obtained with ion-exchange resins. CANALS[11] examined the adsorption of copper and zinc ions on calcium carbonate by chromatographic methods in conjunction with tracer techniques.

Among the studies in this direction should be mentioned the investigations of GAPON[51] with ^{32}P (as phosphate) on alumina, of OLSHANOVA[135] on the adsorption and desorption of cations on alumina, and of KOPYLOVA[82] on the possibilities of separation in precipitation chromatography.

(*m*) *Special analytical methods*

The suitability of alumina for the separation of similar ions has often been investigated. For example, SCHWAB[164] separated the platinum metals and VENTURELLO[203] has also successfully tackled this problem.

The separation of rare earths on alumina was investigated by ERÄMETSA[21,22] and CROATTO[14,15] who were, like LINDNER[93–97] (see above), unable to achieve satisfactory results. However, FISCHER[30a,33] reported that the obtained good results in combination with other methods. HANSEN[61,62] separated zirconium from hafnium on silica; the addition of methanol to the solvent leaves open the question whether it is partition chromatography or molecular adsorption.

As an aid to quantitative determinations, alumina columns were used by DEAN[19] for the removal of fluoride and sulphate, and by NYDAHL[122] for the removal of these ions as well as monohydrogen phosphate ions. VICHUTINSKII[207] removed phosphate on an alumina column in the analysis of plant ashes.

OLSHANOVA[130–134] described qualitative schemes of analysis in which alumina columns played an important part. BISHOP[7] and FILLINGER[28] made suggestions for the introduction of inorganic chromatography in qualitative teaching schemes.

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